

APPLICATION FOR UNITED STATES PATENT

for

COMPOSITIONS AND METHODS FOR MANIPULATING GLIAL PROGENITOR CELLS AND TREATING NEUROLOGICAL DEFICITS

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COMPOSITIONS AND METHODS FOR MANIPULATING GLIAL PROGENITOR CELLS AND TREATING NEUROLOGICAL DEFICITS

Related Applications

5 This application is a continuation-in-part (CIP) of U.S. Patent Application
Serial No. ("USSN") 09/129,028, filed August 04, 1998; which claims the benefit of priority
under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/055,383, filed August 04,
1997. Each of the aforementioned applications are explicitly incorporated herein by
reference in their entirety and for all purposes.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

10 This invention was made in part with Federal Government support under
National Institutes of Health grant nos. NS 15321; NS 0735-5; NS 26761; NS 10175. The
Federal Government may have certain rights in the invention.

TECHNICAL FIELD

15 This invention generally relates to neuroscience and medicine. In particular,
the invention provides compositions and methods for attracting glial and neuronal progenitor
cells and their progeny to desired sites within the central nervous system tissue. These
compositions and methods can also be used to induce directed differentiation of these cells.
By providing various ways to generate new glial and neuronal cells from endogenous
20 progenitor cells, the invention also provides methods for inducing regeneration of tissues and
neurological function, and, indeed, generating new phenotypes and capabilities. Thus, the
invention features methods and compositions for ameliorating neurological deficits,
including inherited disorders, trauma, autoimmune diseases, infections and the like.

BACKGROUND

25 Neurotrophic factors are peptides that variously support the survival,
proliferation, differentiation, size, and function of nerve cells (for review, see, e.g., Loughlin and
Fallon, Neurotrophic Factors, Academic Press, San Diego, CA, 1993). While the numbers of
identified trophic factors, or growth factors, are ever-increasing, most can be assigned to one or
another established family based upon their structure or binding affinities. Growth factors from

various families, including the epidermal growth factor (EGF) family, have been demonstrated to support dopaminergic neurons of the nigrostriatal system (an area that can be treated according to the methods of the present invention) (for review, see, e.g., Hefti, (1994) J. Neurobiol. 25:1418-1435; Unsicker, *Prog. Growth Factor Res.* 5:73-87, 1994).

EGF, the founding member of the EGF family, was characterized more than 25 years ago (Savage and Cohen, *J. Biol. Chem.* 247:7609-7611, 1972; Savage *et al.*, *J. Biol. Chem.* 247:7612-7621, 1972). Since then, additional members have been identified; they include vaccinia virus growth factor (VGF; Ventatesan *et al.*, *J. Virol.* 44:637-646, 1982), myxomavirus growth factor (MGF; Upton *et al.*, *J. Virol.* 61:1271-1275, 1987), Shope fibroma virus growth factor (SFGE; Chang *et al.*, *Mol. Cell. Biol.* 7:535-540, 1987), amphiregulin (AR; Kimura *et al.*, *Nature* 348:257-260, 1990), and heparin-binding EGF-like growth factor (HB-EGF; Higashiyama *et al.*, *Science* 251:936-939, 1991). A common feature of these factors is an amino acid sequence containing six cysteines that form three disulfide cross links and support a conserved structure that underlies their common ability to bind the EGF receptor.

EGF is by far the most-studied member of the family and was the first localized to brain tissue: EGF-like immunoreactivity (IR) was found in areas of developing adult forebrain and midbrain including the globus pallidus, ventral pallidum, entopeduncular nucleus, substantia nigra, and the Islands of Calleja (Fallon (1984) *Science* 224:1107-1109).

Another member of the EGF family, TGF α , has also been localized to brain tissue. It binds the EGF receptor (Todaro *et al.*, *Proc. Natl. Acad. Sci. USA* 77:5258-5262, 1980), stimulates the receptor's tyrosine kinase activity, and elicits similar mitogenic responses in a wide variety of cell types (for review, see Derynck, *Adv. Cancer Res.* 58:27-52, 1992). TGF α might also bind to additional, unidentified receptors (which may help explain its differential actions in some cells). TGF α -IR has previously been shown to be heterogeneously distributed in neuronal perikarya throughout the adult rat CNS and in a subpopulation of forebrain astrocytes (Code *et al.*, *Brain Res.* 421:401-405, 1987; Fallon *et al.*, *Growth Factors* 2:241-250, 1990). TGF α mRNA has been detected in whole rodent brain (Lee *et al.*, *Mol. Cell. Biol.* 5:3655-3646, 1985; Kudlow (1989) *J. Biol. Chem.* 264:3880-3883) and in striatum and other brain regions by a nuclease protection assay (Weickert (1995) *Devel. Brain Res.* 86:203-216) and by *in situ* nucleic acid hybridization (Seroogy *et al.*, *Neuroreport* 6:105-108, 1994). TGF α and EGF mRNAs reach their highest relative abundance (compared to total RNA) in the

early postnatal period and decrease thereafter, suggesting a role in development (Lee *et al.*, 1985, *supra*; Lazar (1992) *J. Neurosci.* 12:1688-1697). In whole brain, the reduction is over 50% (Lazar and Blum, 1992, *supra*), whereas, in striatum, relative TGF α mRNA drops by two-thirds from peak levels (Weickert and Blum, 1995, *supra*). At all developmental stages examined, whole brain TGF α mRNA exceeds EGF mRNA levels by more than an order of magnitude (Lazar (1992) *supra*).

The EGF receptor was localized by immunocytochemistry to astrocytes and subpopulations of cortical and cerebellar neurons in rat brain and to neurons in human autopsy brain specimens (Gomez-Pinilla *et al.*, *Brain Res.* 438:385-390, 1988; Werner *et al.*, *J. Histochem. Cytochem.* 36:81-86, 1988). EGF binding sites were revealed in rat cortical and subcortical areas, including the striatum, in an autoradiography study with radiolabeled EGF (Quirion *et al.*, *Synapse* 2:212-218, 1988). *In situ* hybridization studies demonstrated EGF receptor mRNA in striatum and cells of the ventral mesencephalon (Seroogy *et al.*, 1994, *supra*) and in proliferative regions in developing and adult rat brain (Seroogy *et al.*, *Brain Res.* 670:157-164, 1995). As with relative EGF and TGF α mRNAs, EGF receptor mRNA is most abundant in striatum and ventral midbrain early in development, and gradually declines as the animal matures (Seroogy *et al.*, 1994, *supra*).

Physiologically, TGF α acts on numerous cell types throughout the body, including many of neural origin (for review, see Derynck, 1992, *supra*). It supports the survival of cultured central neurons (Morrison *et al.*, *Science* 238:72-75, 1987; Zhang *et al.*, *Cell. Regul.* 1:511-521, 1990) and, unlike EGF, enhances survival and neurite outgrowth of dorsal root ganglion sensory neurons (Chalazonitis *et al.*, *J. Neurosci.* 12:583-594, 1992). It also stimulates proliferation and differentiation of neuronal and glial progenitor cells from developing and adult brains (Anchan *et al.*, *Neuron* 6:923-936, 1991).

The trophic effects of EGF-family peptides on mesencephalic dopaminergic neurons in culture have also been studied in recent years. EGF enhances the survival of E16 dopamine neurons in mixed midbrain cultures (Casper *et al.*, *J. Neurosci. Res.* 30:372-381, 1991), but the degree to which it stimulates dopamine uptake is modest (Knusel *et al.*, *J. Neurosci.* 10:558-570, 1990). TGF α also supports the survival of mesencephalic dopamine neurons in dissociated cell culture, but its effect is more selective than that of EGF (Ferrari *et al.*, *J. Neurosci. Res.* 30:493-497, 1991; Alexi and Hefti, *Neurosci.* 55:903-918, 1993).

Another important characteristic of EGF-family growth factors is their ability to protect midbrain dopamine cells from neurotoxic assaults. EGF has been shown to protect dopamine neurons from glutamate or quisqualate excitotoxicity in dissociated cell culture (Casper (1995) *J. Neurochem.* 65:1016-1026). It has also been demonstrated to protect cultured dopamine cells from the selective dopamine neurotoxin, 1-methyl-4-phenylpyridinium (MPP⁺; Park (1992) *Brain Res.* 599:83-97) and to increase dopamine uptake in MPP⁺-treated cultures (Hadjiconstantinou (1991) *J. Neurochem.* 57:479-482).

The results of studies with EGF *in vivo* were consistent those obtained in culture; EGF effected neuroprotection in both instances. For example, intracerebro-ventricular (ICV) infusions of EGF reduced amphetamine-induced rotations, increased the number of surviving tyrosine hydroxylase immunoreactive (TH-IR) cells in the SN, and increased striatal TH-IR fibers after transection of the nigrostriatal pathway in a rat model of PD (Pezzoli *et al.*, *Movement Disord.* 6:281-287, 1993; Ventrella, *J. Neurosurg. Sci.* 37:1-8, 1993). ICV infusions of EGF into the brains of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mice enhanced the content of dopamine and 3,4-dihydro-zyphenylacetic acid (DOPAC) and the activity of tyrosine hydroxylase in the striatum (Hadjiconstantinou (1991) *supra*; Schneider *et al.*, *Brain Res.* 674:260-264, 1995).

Despite its more potent activity *in vitro*, relative to EGF, the trophic effects of TGF α *in vivo* - particularly in animals, including humans, with neurological deficits - are undetermined.

SUMMARY

The invention provides compositions and methods for attracting glial and neuronal progenitor cells and their progeny to desired sites within the central nervous system (CNS) tissue. The methods comprise administration of a compound that binds to an epidermal growth factor (EGF)/ErbB family receptor (i.e., an ErbB family receptor ligand) to a site in the central nervous system. These compositions and methods can also be used to induce directed differentiation of glial and neuronal progenitor cells. By providing various ways to generate new glial and neuronal cells from endogenous progenitor cells, the invention also provides methods for inducing regeneration of tissues and neurological function, and, indeed, generating new phenotypes and capabilities. Because glial cells can act as a guide for the movement of neuronal cells, manipulating the migration and

differentiation of glial cells can aid in the "design" of new *in vivo* generated neuronal cells tissues and organs. For example, as described below, in some developmental processes, migrating neuroblasts are sheathed by differentiated glial cells, such as astrocytes, which border the neuroblasts to create a highly restricted migratory pathway. The migrating
5 neuronal cells essentially form a solid stream of moving cells within a tube of specialized glial guide cells. Thus, the invention features methods and compositions for generating, or regenerating, neuronal tissues and their functions *in vivo* and for ameliorating neurological deficits, including inherited disorders, trauma, infections and the like.

The invention provides a method for attracting a glial progenitor cell, or a
10 progeny of a glial progenitor cell, to a site in a central nervous system (CNS) tissue, the method comprising the steps of: (a) providing a compound that binds to an epidermal growth factor (EGF)/ErbB family receptor; and, (b) administering a sufficient amount of the compound to the site to attract the glial progenitor cell or progeny thereof to the site in the central nervous system (CNS) tissue. The method can further comprises administering a
15 sufficient amount of the compound to stimulate differentiation of the glial progenitor cell or progeny thereof.

In one aspect of the method, the compound is administered *in vivo*. The compound can be administered by intracerebroventricular infusion or by intrastriatal infusion. The compound can be administered into a brain tissue, such as a tissue adjacent to
20 a subependymal zone. Alternatively, the compound can be administered into spinal cord tissue. Alternatively, the compound can be administered to a single cell, or a tissue or an organ culture environment comprising a glial progenitor cell.

In alternative aspects of the method, the administered compound comprises an amphiregulin (AR), a betacellulin (BTC), an ephrine (ER), a heparin-binding EGF-like
25 growth factor (HB-EGF), a schwannoma-derived growth factor (SDGF), a myxomavirus growth factor Shope fibroma virus growth factor, a teratocarcinoma-derived growth factor-1 (TDGF-1), a transforming growth factor alpha (TGF α), heregulin (HRG), neuregulin-3, or a vaccinia growth factor (VGF), or equivalents thereof, or a combination thereof.

In one aspect, the method further comprises administering a second
30 compound. The second compound can be capable of increasing the expression of a cell adhesion molecule or extracellular matrix molecule. The second compound can be

administered along or at the end of a desired path of migration of the glial progenitor cell or its progeny thereof. The second compound can stimulate expression of extracellular matrix or cell adhesion molecules. The second compound can be a transforming growth factor beta (TGF- β). The cell adhesion molecule can be fibronectin. The cell adhesion molecule can be laminin. The second compound can be capable of inhibiting a naturally occurring signal that would otherwise inhibit migration of the glial progenitor cell or its progeny. In the methods of the invention, the first or second administered compound can be applied along a path between a glial precursor cell and a site toward which it is desired that the progeny migrate.

The methods of the invention can further comprise mechanically disrupting tissue in the central nervous system (CNS), thereby directing migration of a glial progenitor cell or its progeny. The also can further comprise neurochemically blocking the activity of the central nervous system (CNS) tissue, thereby directing migration of glial progenitor cell or its progeny.

The methods of the invention can further comprise administering a second compound, wherein the second compound is capable of stimulating differentiation in the glial progenitor cell or its progeny. The differentiation-stimulating compound can comprise a retinoic acid or a brain-derived neurotrophic factor.

The invention provides a method for attracting a glial progenitor cell, or a progeny thereof, to a site in a central nervous system (CNS) tissue comprising administering a sufficient amount of transforming growth factor alpha (TGF α) polypeptide, or functional fragment thereof, to the site to attract the glial progenitor cell or its progeny to the site.

The invention provides a method for ameliorating a neurological deficit in a patient, the method comprising administering to a central nervous system (CNS) tissue of the patient a compound that binds to an epidermal growth factor (EGF) /ErbB family receptor, wherein the administration stimulates the proliferation of a glial progenitor cell or its progeny, thereby ameliorating the neurological deficit. The method can further comprise administering a sufficient amount of the compound to direct the migration of the glial progenitor cell or its progeny a desired site in the central nervous system (CNS). The method can further comprise administering a sufficient amount of a second compound to stimulate the differentiation of the glial progenitor cell or its progeny to the site.

In alternative aspects, the neurological deficit is caused by a neurodegenerative disease, a neurotoxic injury, a developmental disorder, a disorder affecting vision, audition, somatosensation or olfaction, a disease of the spinal cord, an autoimmune disease, an infection, or an inflammatory disease. The neurological deficit can be a traumatic injury, such as an injury of the spinal cord. The neurological deficit can be a demyelinating disease. The neurological deficit can be Alzheimer's Disease. The neurological deficit can be Huntington's Disease. In one aspect, the neurological deficit can be Parkinson's Disease. Administration of the compound can results in differentiation of striatal cells into dopamine-producing cells. In one aspect, the neurological deficit is an ischemia. The ischemia can be associated with a stroke.

The invention provides a method for ameliorating a neurological deficit in a patient, the method comprising administering a first compound to a first site in a patient's central nervous system (CNS), wherein the first compound is capable of binding to an epidermal growth factor (EGF) /ErbB family receptor, with the proviso that the compound is not an EGF polypeptide, wherein the binding of the first compound to the EGR receptor results in signal transduction sufficient to stimulate the proliferation of the glial progenitor cell and its progeny and to direct migration of the glial progenitor cell and its progeny to a second site in the CNS, thereby ameliorating the neurological deficit. In one aspect, the method further comprises administering a second compound, wherein the second compound is capable of stimulating differentiation of the glial progenitor cell or its progeny. The first compound and the second compound can be administered in any order, for example, they can be simultaneously or sequentially administered.

The invention provides a pharmaceutical composition comprising a compound and a pharmaceutically acceptable carrier, wherein the compound comprises a first agent capable of binding to an epidermal growth factor (EGF) /ErbB family receptor and a second agent capable of stimulating the differentiation of glial progenitor cells. In one aspect of the pharmaceutical composition of the invention, the first agent can comprise a polypeptide that binds the EGF receptor. The polypeptide can be a transforming growth factor alpha (TGF α) polypeptide, a functional variant thereof, or a biologically functional equivalent thereof. The second agent can comprise a brain-derived neurotrophic factor polypeptide or a retinoic acid.

The invention provides a method for ameliorating a neurological deficit, the method comprising contacting a glial progenitor cell of the patient's central nervous system (CNS) with a transforming growth factor alpha (TGF α) polypeptide, a functional variant thereof, or a biologically functional equivalent thereof, that binds to an epidermal growth factor (EGF) /ErbB family receptor expressed by the glial progenitor cell, wherein the binding of the TGF α polypeptide to the EGF receptor results in a signal transduction sufficient to stimulate the proliferation of the glial progenitor cell and to direct migration of the progeny of the proliferating glial progenitor cell to a region of the CNS in which the progeny cells can function in a manner sufficient to ameliorate the neurological deficit.

The invention provides a method for ameliorating a neurological deficit, the method comprising contacting a glial progenitor cell of the patient's central nervous system (CNS) with a polypeptide that binds to an epidermal growth factor (EGF) /ErbB family polypeptide family receptor to which a transforming growth factor alpha (TGF α) polypeptide, a functional variant thereof, or a biologically functional equivalent thereof can bind, with the proviso that the polypeptide is not an EGF polypeptide, wherein the binding of the polypeptide to the EGF polypeptide family receptor results in a signal transduction sufficient to stimulate the proliferation of the glial progenitor cell and to direct migration of the progeny of the glial progenitor cell to a region of the CNS in which the progeny cells can function in a manner sufficient to reduce the neurological deficit.

The present invention is, in part, based on newly discovered effects of TGF α infusion on cells in the normal and abnormal (lesioned) central nervous system.

The present invention features methods and compositions for treating a patient who has a neurological deficit. The method can be carried out, for example, by contacting (*in vivo* or in culture) a neural or glial progenitor cell of the patient's central nervous system (CNS) with a polypeptide that binds the epidermal growth factor (EGF) /ErbB family receptor and directing progeny of the proliferating progenitor cells to migrate *en masse* to a region of the CNS in which they will reside and function in a manner sufficient to reduce the neurological deficit. The method may include a further step in which the progeny of the neural or glial precursor cells are contacted with a compound that stimulates differentiation.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

Fig. 1 is a schematic of a coronal section of rat forebrain. The injection pipette illustrated on the right-hand side represents one location into which a growth factor can be infused, the striatum (str). (cerebral cortex = ctx; lateral ventricle = lv).

Figs. 2A-2D are photomicrographs of coronal sections through the mesencephalon of the adult rat brain that have been probed to show the distribution of EGF receptor mRNA. In Fig. 2A a "sense" probe was applied as a control. In Figs. 2B-2D, sections through the substantia nigra (sn) reveal moderately abundant expression in the hippocampus (hip), the medial portion of the sna, and the parabrachial and paranigral nuclei of the ventral tegmental area (vta). In the most-caudal midbrain (Fig. 2D), the interpeduncular nucleus (ip) was the most intensely labeled. Scale bar = 5 mm.

Fig. 3 is a photomicrograph of a coronal section through the striatum of an adult rat brain that was infused with TGF α and probed to show the distribution of EGF receptor mRNA. On the side of the infusion, a dramatic increase in hybridization density is apparent in the medial striatum adjacent to the lateral ventricle.

Fig. 4 is a photomicrograph of a coronal section through the forebrain of an adult rat brain that was infused with TGF α and lesioned with 6-OHDA. *In situ* hybridization was performed to localize EGF receptor mRNA, which appears as an intense ridge extending well into the body of the striatum.

Fig. 5 is a bar graph showing the average standardized densities of TGF α mRNA hybridization in striata in each of five groups of animals examined (normal; artificial cerebrospinal fluid ("aCSF") infusion, no lesion; aCSF infusion, lesion; TGF α infusion, no lesion; TGF α infusion, lesion). The paired bars represent densities in striata ipsilateral and contralateral to the treatment. Average hybridization density was significantly reduced by one-quarter ipsilateral to the treatments in both groups receiving nigral 6-OHDA lesions. The striatal infusion of TGF α peptide had no impact on the decrease. Averages \pm S.E.M.

(Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.005$, ** $p < 0.001$).

Fig. 6 is a bar graph showing the average standardized densities of EGF receptor mRNA hybridization in the subependymal regions along the edges of the striata bordering the lateral ventricles of animals in the same five test groups described in Fig. 5. The paired bars represent densities in striata ipsilateral and contralateral to the treatment. Average hybridization density was approximately doubled in the ipsilateral subependymal region in both groups receiving TGF α striatal infusions. Averages \pm S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.01$, ** $p < 0.0001$).

Fig. 7 is a bar graph showing the average standardized densities of EGF receptor mRNA hybridization in the striatal ridges, the non-ridge body of the striatum, and the subependymal regions in all animals with striatal ridges. The paired bars represent densities in striata ipsilateral and contralateral to the treatment. Average hybridization density was highest in the striatal ridge ipsilateral to the treatments. No striatal ridges ever appeared in the contralateral striata. TGF α striatal infusions. Averages \pm S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.005$, ** $p < 0.001$).

Figs. 8A and 8B are photomicrographs of coronal sections through the striatum of an adult rat that received a nigral 6-OHDA lesion and TGF α infusion for fourteen days. In Fig. 8A, silver staining in the caudate-putamen ipsilateral to the treatments reveals huge numbers of cells in the dorsal portion of the ridge, many of which exhibit an elongated morphology and are oriented normal to the subependymal region. There is also an increase in the number of cells along the lateral ventricle (lv). In the contralateral striatum (Fig. 8B), the cellular population is not expanded, either in the striatum or along the lateral ventricle.

Figs. 9A-9D are photomicrographs of thionin-stained coronal sections of adult rat brain from animals that were lesioned with 6-OHDA and infused with TGF α for variable periods of time. In Fig. 9A, after four days of infusion, cellular expansion in the subependymal region is barely detectable above background staining. In Fig. 9B, after six days of infusion, the aggregation of thionin-stained cells near the lateral ventricle is much more robust. In Fig. 9C, after nine days of infusion, a region of densely-stained cells appears slightly lateral to the subependymal zone at the ventral end of the cellular expansion. In

Fig. 9D, after fourteen days of infusion, a dense, well-formed ridge is evident well into the body of the striatum.

Figs. 10A and 10B are photomicrographs of coronal sections of adult rat brain from animals that were lesioned with 6-OHDA and infused with TGF α for fourteen days. In Fig. 10A, nestin immunohistochemistry reveals an intense striatal ridge. In Fig. 10B, thionin staining of a near-adjacent section confirms the registry between the nestin-IR cells and the striatal ridge.

Figs. 11A-11C are photomicrographs of thionin stained coronal sections from adult rat brain from animals that were lesioned with 6-OHDA and infused with TGF α , at varying distances from the lateral ventricle, for fourteen days. In Fig. 11A, where the infusion cannula was implanted in the far-lateral striatum, the ridge parallels the subependymal zone and is less dense than that seen with a mid-striatal infusion. In Fig. 11B, where the infusion cannula was implanted mid-striatum, the ridge is characteristically S-shaped, with the ventral portion extending far out into the ventral striatum. In Fig. 11C, where the infusion was immediately adjacent to the lateral ventricle, the striatal ridge is L-shaped and generally exhibits very dense thionin staining.

Fig. 12 is a bar graph depicting the maximum displacement of the striatal ridge (from the lateral ventricle; in mm) in coronal sections of adult rat brain after nigral lesion with 6-OHDA and mid-striatal infusion of TGF α for fourteen days. Animals treated according to "Schedule A" (left-most bar) received lesions first, followed by TGF α infusions four to five weeks later. Animals treated according to "Schedule B" received lesions two days after the 14-day TGF α infusion began. Averages \pm S.E.M. (Student's *t*-test; P value, * $p < 0.01$).

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention provides an *in vivo* procedure for the induction of massive proliferation, directed migration, and neurodifferentiation ("PMD") in the damaged adult central nervous system. The methods of the invention can be used for the amelioration and treatment of human neurological deficits, e.g., neurodegenerative disorders, such as Parkinson's Disease.

To demonstrate this ability, *in vivo* induction of proliferation, directed migration, and neurodifferentiation in the forebrain of the adult rat was demonstrated using a combination

of a 6-hydroxydopamine lesion of the substantia nigra dopaminergic neurons and an exemplary embodiment of the invention comprising infusion of transforming growth factor alpha (TGF α) into forebrain structures, as discussed in detail in Example 3, below. In animals with both induced lesion and infusion of TGF α , was there a rapid proliferation of forebrain stem cells followed by a timed migration of a ridge of neuronal and glial progenitors directed toward the region of the TGF α infusion site. Subsequently, increasing numbers of differentiated neurons were observed in the striatum. In behavioral experiments, there was a significant reduction of apomorphine-induced rotations in animals receiving the TGF α infusions. These results show, for the first time, that the brain contains stem cells capable of proliferation, directed migration, and neurodifferentiation in response to an exogenously administered growth factor. Accordingly, the methods of the invention can be used in the amelioration and treatment of both acute neural trauma and neurodegenerative diseases.

While the invention is not limited by any particular mechanism of action, it may be that the embryonic germinal layer of the central nervous system, e.g., that which is retained in an adult subventricular zone (SVZ) throughout the neuraxis, is responsive to the microenvironmental signals generated by infusion of compounds that bind to epidermal growth factor (EGF)/ ErbB family receptors expressed by neuronal and glial progenitor cells, such as TGF alpha and the other ErbB receptor ligands described herein. The result is proliferation, "directed" migration, maturation and differentiation of newly generated neuronal and glial cells.

The invention provides methods for attracting neuronal and glial progenitor cells, or progeny thereof, to a site in a central nervous system (CNS) tissue, the method comprising the steps of: (a) providing a compound that binds to an epidermal growth factor (EGF)/ ErbB family receptor (i.e., an ErbB family receptor ligand); and, (b) administering a sufficient amount of the compound to the site to attract the neuronal and/or glial progenitor cell or progeny thereof to the site in the central nervous system (CNS) tissue. The epidermal growth factor (EGF)/ErbB family of receptors (also called the "ErbB tyrosine kinase receptor family") mediate a variety of cellular responses to epidermal growth factor (EGF)-like growth factors. Six exemplary ErbB receptor polypeptide ligands that can be administered in practicing the methods of the invention include, e.g., epidermal growth factor (EGF) (see, e.g., U.S. Patent Nos. 5,158,935; 4,743,679; 4,717,717; 4,686,283; 4,621,052), transforming growth factor-alpha (TGF-alpha) (see, e.g., U.S. Patent Nos. 5,190,858; 5,182,261), betacellulin (BTC), heparin-

binding epidermal growth factor-like growth factor (HB-EGF) (see, e.g., U.S. Patent No. 6,156,522), amphiregulin (AR) (see, e.g., U.S. Patent No. 6,071,889), heregulin (HRG) (see, e.g., U.S. Patent Nos. 6,136,558; 5,968,511; 5,367,060; 5,641,869), neuregulin-3 (see, e.g., U.S. Patent No. 6,121,415). See, e.g., Sweeney (2000) *Oncogene* 19:5568 - 5573; O-Charoenrat (2000) *Int. J. Cancer* 88:759-765; Zheng (1999) *J. Neurocytol.* 28:901-912; Ojeda (1999) *J. Neurobiol.* 40:528-540; Eagleson (1998) *Mol. Cell. Neurosci.* 12:349-362.

ErbB receptor polypeptide ligands that can be administered in practicing the methods of the invention can also include receptor-binding peptides based on natural ligands (see, e.g., U.S. Patent No. 5,874,528). ErbB receptor ligands that can be administered also include "analogs," or "conservative variants" and "mimetics" (e.g., "peptidomimetics") with structures and activity that substantially correspond to the polypeptides used in practicing the methods of the invention, e.g., ErbB receptor polypeptide ligands. Thus, the terms "conservative variant" or "analog" or "mimetic" also refer to a polypeptide or peptide which has a modified amino acid sequence, such that the change(s) do not substantially alter the polypeptide's (the conservative variant's) structure and/or activity (e.g., binding specificity to ErbB family receptors expressed on neural and glial progenitor cells). These include conservatively modified variations of an amino acid sequence, i.e., amino acid substitutions, additions or deletions of those residues that are not critical for protein activity, or substitution of amino acids with residues having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids does not substantially alter structure and/or activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary substitution): ala/gly or ser; arg/lys; asn/gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gln or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu. An alternative exemplary guideline uses the following six groups, each containing amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); (see also, e.g., Creighton (1984) *Proteins*, W.H. Freeman

and Company; Schulz and Schimer (1979) Principles of Protein Structure, Springer-Verlag). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, for some purposes, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be considered "conservatively modified variations."

The terms "mimetic" and "peptidomimetic" refer to a synthetic chemical compound that has substantially the same structural and/or functional characteristics of the polypeptides of the invention (e.g., ability to bind ErbB receptors on neural and glial progenitor cells). The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetics' structure and/or activity. As with polypeptides of the invention which are conservative variants, routine experimentation will determine whether a mimetic is within the scope of the invention, i.e., that its structure and/or function is not substantially altered. Polypeptide mimetic compositions can contain any combination of non-natural structural components, which are typically from three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. A polypeptide can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclo-hexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., -C(=O)-CH₂- for -C(=O)-NH-), aminomethylene (CH₂-NH), ethylene, olefin (CH=CH), ether (CH₂-O), thioether (CH₂-S), tetrazole (CN₄-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and

Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY). A polypeptide can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues; non-natural residues are well described in the scientific and patent literature.

5 ErbB tyrosine kinase receptor family members, and screening methods for identifying ErbB family receptor members and ErbB receptor ligands, are described, e.g., in U.S. Patent Nos. 6,040,290; 5,985,553; 5,916,755; 5,910,583; 5,874,528; 5,587,458; 5,578,482; 5,480,968.

I. The Striatum and Nigrostriatal System

A. Anatomy, Connectivity and Neurochemistry

10 Within the brain, the striatum, pallidum, substantia nigra, ventral tegmental area (VTA), subthalamic nucleus, and amygdala are collectively referred to as the basal ganglia. The striatum contains dorsal and ventral components, each of which is further subdivided into additional anatomical structures. In humans, the dorsal striatum consists of
15 the caudate nucleus and the putamen. The C-shaped caudate follows the curve of the lateral ventricles. Its tail portions extend past the ends of the inferior horns and joins the amygdala in each temporal lobe. The head of the caudate turns ventrally from the anterior end of the anterior horns and fuses with the putamen. Although largely anatomically distinct in the human brain, they are combined into a common structure, the caudate-putamen or
20 caudoputamen, in rodents.

The ventral striatum is comprised of the nucleus accumbens, olfactory tubercle, and the associated striatal cell bridges. The pallidum includes the globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata (SNr), and the ventral pallidum. The entopeduncular nucleus and SNr have very similar afferent and efferent connections. The
25 ventral pallidum contains regions that have a mix of connections that are similar to both the globus pallidus and entopeduncular nucleus. The other part of the substantia nigra, the pars compacta (SNc), includes dopamine neurons that span the substantia nigra-ventral tegmental area (SN-VTA), as well as dopamine cell clusters in the SNr. The circuitry of the basal ganglia is complex, but is very similar in both rats and humans (Fallon and Loughlin,
30 Cerebral Cortex, E.G. Jones and A. Peters, Eds., Vol. 6, pp. 41-127, Plenum Press, New York, 1987; Alheid and Heimer, *Progr. Brain Res.* 107:461-484, 1996), making the rat a

useful model for studying connections, neurochemistry, pharmacology, function, and clinical correlates of this system in the mammalian brain.

The striatum, together with other nuclei of the basal ganglia, contributes to the regulation of movement and emotion. A number of diseases affecting the system or its innervation are associated with profoundly debilitating motor impairment, often accompanied by affective disorders.

The caudate and putamen are the primary input nuclei of the basal ganglia and receive major excitatory projections from the cerebral cortex and the centromedian and intralaminar nuclei of the thalamus. Corticostriatal afferents are glutamatergic. Afferents from the thalamus are also thought to be glutamatergic. The substantia nigra pars compacta (SNc) provides dense dopaminergic input to the striatum via the nigrostriatal pathway (for review of this system in the rat, see Fallon and Loughlin, The Rat Nervous System, G. Paxinos, Ed., pp. 215-237, Academic Press, San Diego, 1995). The ventral striatum and nucleus accumbens receive the bulk of their dopaminergic innervation from dopamine cells of the VTA in the ventromedial mesencephalon. Limbic afferents from the amygdala and serotonergic fibers from the midbrain or raphe also terminate in the ventral striatum.

The distribution of striatal afferents and their terminations are not simply uniform representations of their regions of origin. The striatum is organized into patches or striosomes embedded in a functionally and chemically distinct surrounding matrix. This organization was originally demonstrated using histochemistry for acetylcholinesterase (AChE), which selectively stains the matrix (Graybiel and Ragsdale, *Proc. Natl. Acad. Sci. USA*, 75:5723-5726, 1978). Since then, enzyme histochemistry, immunocytochemistry, *in situ* hybridization, receptor binding with radiolabeled ligands, anterograde degeneration and other methods have been used to identify many additional markers that are differentially distributed in the two compartments. Markers for the matrix include calbindin, somatostatin, and dopamine uptake ($[^3\text{H}]$ mazindol binding) sites (Gerfen, *J. Comp. Neurol.* 236:454-476, 1985; Voorn *et al.*, *J. Comp. Neurol.* 289:189-201, 1989). Striosomes can be identified by their higher relative abundance of enkephalin, 5'-nucleotidase activity with nigrostriatal lesions, tyrosine hydroxylase, mu opoid receptor binding, and substance P (Graybiel *et al.*, *Neurosci.* 6:377-397, 1981; Schoen and Graybiel, *J. Comp. Neurol.* 322:566-576, 1992). As

might be expected, however, there are interspecific and developmental variations in many of these markers and some are useful only in certain regions of the striatum.

The heterogeneity of chemical markers is further complicated by the selective origin and termination of many striatal pathways in the patch or matrix compartments. For instance, afferents from motor, cingulate, somatosensory and visual areas of cortex terminate in the matrix (Gerfen, *Nature* 311:461-464, 1984; Donoghue and Herkenham, *Brain Res.* 365:397-403, 1986). The bulk of the corticostriatal afferents from deep layer V and layer VI of limbic cortex terminate in the patches while most from more superficial layer V and layers II and III provide input to the matrix (Gerfen, *Science* 246:385-388, 1989). Afferents from the VTA and the dorsal tier of the SNc provide dopaminergic input to the matrix. The patches receive dopamine innervation from the ventral tier of the SNc and dopamine cell clusters in the SNr (Schoen and Graybiel, *J. Comp. Neurol.* 322:566-576, 1992). In the dorsal striatum, inputs from nuclei in the medial division of the thalamus terminate in the patches while afferents from the lateral division - including anterior and posterior intralaminar and rostral ventral tier nuclei - predominantly innervate matrix tissue (Ragsdale and Graybiel, *J. Comp. Neurol.* 311:134-167, 1991). In addition, amygdalostriatal fibers originating in the basolateral nucleus of the amygdala selectively innervate the patch compartment (Ragsdale and Graybiel, *J. Comp. Neurol.* 269:506-522, 1988).

Striatal efferents are also differentially distributed with respect to the patch-matrix organization. The striatonigral pathway, one of the two major pathways originating in the striatum, has been shown to be comprised of two distinct projections. Fibers arising from neurons in the patch compartment terminate around dopamine neurons in the ventral SNc and in dopamine cell clusters in the SNr. Matrix neurons give rise to topographically-arranged projections to the SNr, including non-dopaminergic areas and dopamine neurons whose dendrites are located in the SNr (Gerfen, 1984, *supra*; Jiminez-Castallanos and Graybiel, *Neurosci.* 32:297-321, 1989).

The other major efferent projection, the striatopallidal pathway, projects to the globus pallidus. It has not been shown to be distributed with respect to the patch-matrix organization; however, it is neurochemically distinct from the striatonigral system. The majority of striatopallidal fibers express enkephalin and not dynorphin or substance P. In contrast, few striatonigral projections contain enkephalin, but most express dynorphin and

substance P (Gerfen and Young, *Brain Res.* 460:161-167, 1988). In primates, the two systems also differ in their anatomical regions of origin: striatopallidal efferents arise mainly from the putamen while striatonigral efferents originate primarily in the caudate (Parent *et al.*, *Brain Res.* 303:385-390, 1984).

In addition to the heterogeneous distribution of striatal connections, several morphologically and chemically distinct types of neurons are found in the striatum (Albin *et al.*, *Trends Neurosci.* 12:366-375, 1989; Groves, *Brain Res. Rev.* 5:234-238, 1983). They are traditionally classified as either spiny or aspiny based on their dendritic morphology. There are two generally recognized types of spiny neurons in the striatum. They contain various combinations of GABA, substance P, enkephalin and dynorphin, but are predominantly GABAergic. The medium spiny neurons (spiny type I) are by far the most abundant, comprising 90-95% of all striatal neurons. They have smooth cell bodies and dense accumulations of spines on the distant portions of their dendrites. Their dendritic arborizations range to about 200 μ m from the somata. Medium spiny neurons are the principle terminal targets for dopaminergic neurons in the SNc, which form synapses predominantly on the necks of the dendritic spines. Spiny type II neurons are much larger, with variable arbors extending up to about 600 μ m from the soma.

Spiny neurons are the projection neurons of the striatum. Those in the matrix containing GABA and substance P project predominantly to the internal segment of the globus pallidus (GP_i) and the SNr. Spiny GABAergic matrix neurons containing enkephalin, on the other hand, innervate the external segment of the globus pallidus (GP_e). Spiny neurons in the patch compartment send the majority of their efferents to the SNc (Albin *et al.*, 1989, *supra*).

Striatal projection neurons of the two major efferent pathways can also be distinguished by their dopamine receptor subtypes. Substance P/dynorphin neurons projecting to the substantia nigra express predominantly D₁ dopamine receptors, while enkephalinergic striatopallidal neurons express mainly D₂ receptors. Neither receptor type, however, is expressed exclusively in either projection (Besson *et al.*, *Neurosci.* 26:101-119, 1989; Gerfen *et al.*, *Science* 250:1429-1432, 1990).

Three recognized types of aspiny neurons make up the population of striatal interneurons (Groves, 1983, *supra*; Carpenter, In: Core Text of Neurosciences, pp. 325-360,

Williams and Wilkins, Baltimore, 1991). Together, they make up 10% or less of the total number of neurons of the striatum. Aspiny type I neurons are the most common of the three and have smooth dendrites in arbors slightly smaller than those of medium spiny neurons. They are largely GABAergic, but many contain somatostatin and neuropeptide Y. Aspiny type II neurons are distinguished by their large cell bodies and AChE and choline acetyltransferase (ChAT) staining. This cell type forms symmetric synapses with medium spiny neurons. Medium aspiny type III neurons are the least well characterized but are thought to contain GABA. There are probably additional chemically-defined and connectionally-defined subsets of these classes of neurons beyond the ones already identified.

B. Topography and Development

Experiments with anterograde and retrograde tracers in striatal projections of the mesencephalic dopamine system revealed precise topographies in adult rodents (Fallon and Moore, *J. Comp. Neurol.* 180:545-580, 1978). The dorsal striatum receives dopaminergic innervation from neurons in the ventral and intermediate SN and VTA. The ventral striatum and nucleus accumbens receive dopaminergic input from the dorsal VTA and intermediate SN (Fallon, *Ann. NY Acad. Sci.* 537:1-9, 1988).

Neurogenetic gradients in the developing system parallel the topographic arrangements of projections in the mature system. The dorsolateral portion of the SN is the earliest produced in the embryo, before embryonic day 15 (E15) in the rat (Altman and Bayer, *J. Comp. Neurol.* 198:677-716, 1981). Projections from this region innervate the lateral and ventral regions of the striatum (Carter and Fibiger, *Neurosci.* 2:569-576, 1977; Veening *et al.*, *Neurosci.* 5:1253-1268, 1980), which are also the earliest striatal areas generated (Bayer, *Neurosci.* 4:251-271, 1984). As the striatum is populated with younger neurons in a ventrolateral-to-dorsomedial gradient, afferents arrive from more ventromedial (and later-produced) portions of the SN (generated after E15) to innervate these later-produced striatal areas. Thus, the youngest (ventromedial) nigral dopamine neurons innervate the youngest (dorsomedial) striatal neurons, and older (dorsolateral) nigral dopamine neurons innervate older (ventrolateral) striatal neurons. This pattern is repeated in the GABAergic striatonigral projections as well (Bunney and Aghajanian, *Brain Res.* 117:423-435, 1976).

The neurons of the striatum are derived from a neuroepithelium surrounding the lateral ventricles in the prenatal and early postnatal brain. A ventricular zone initially lines the ventricles, later joined by the subventricular (or subependymal) zone just deep to it. As the brain matures, the ventricular zone disappears, but the subependymal zone persists as a thin layer of cells. This zone contains neural stem cells and progenitor cells that migrate along a defined and restricted path to replenish the labile interneuronal cell population of the olfactory bulb (Luskin, *Neuron* 11:173-189, 1993; Lois and Alvarez-Buylla, *Science* 264:1145-1148, 1994).

C. Pathology

The striatum and its dopaminergic innervation are vulnerable to a number of conditions including several neurodegenerative diseases (Albin *et al.*, 1989, *supra*) such as Huntington's disease (HD) and Parkinson's disease (PD). HD is an autosomal dominant hereditary disease (chromosome 4) characterized by progressive degeneration of the striatum. It is associated with involuntary choreathetotic movements of the limbs and face and disruptions of voluntary movement (for review, see Purdon *et al.*, *J. Psychiatr. Neurosci.* 16:359-367, 1994). Medium spiny GABAergic neurons in the matrix compartment are most affected, especially the GABA/enkephalinergic neurons projecting to the GP_e (Albin *et al.*, 1989, *supra*). Large aspiny cholinergic interneurons and small aspiny interneurons containing somatostatin, neuropeptide Y and NADPH-diaphorase, also found in the matrix compartment, are relatively spared (Ferrante *et al.*, *Science* 230:561-563, 1985; Reiner *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5733-5737, 1988). In more advanced stages of HD, however, neuronal degeneration includes all types of striatal neurons and extends to other nuclei of the basal ganglia, the cerebral cortex, hypothalamus, and cerebellum.

Parkinson's Disease (PD) is characterized by resting tremor, rigidity, inability to initiate movement (akinesia) and slowness of movement (bradykinesia) (Marsden, *Lancet* 335:948-952, 1990). The motor deficits are associated with progressive degeneration of the dopaminergic nigrostriatal pathway and, to various extents, loss of dopaminergic innervation to the nucleus accumbens and degeneration of noradrenergic cells of the locus ceruleus and serotonergic neurons of the raphe (Javoy-Agid *et al.*, *Adv. Neurol.* 40:189-198, 1984; Agid, *Lancet* 337:1321-1327, 1991). Up to 80% of nigral dopamine neurons can be lost before significant motor deficits are manifest.

One of the major strategies for using peptides known as neurotrophic factors as therapeutic agents in the treatment of neurodegenerative diseases is to arrest the degenerative process and enhance the function of remaining cells. The studies presented in the Examples below will illustrate how the present invention expands the use of neurotrophic factors beyond that which has been previously suggested.

II. Treatment of Neurological Deficits

As demonstrated by the examples below, neural precursors in the adult forebrain subependymal zone can be stimulated to proliferate and migrate *en masse* into the central nervous system (CNS) (*e.g.*, into the striatum) in response to an infusion of a polypeptide that binds the EGF receptor (*e.g.*, TGF α ; the term "polypeptide" as used herein refers to any chain of amino acid residues, regardless of length or post-translational modification). Furthermore, one can direct migration of the proliferating cells as a dense ridge. As described below, directed migration can be accomplished in a variety of ways. For example, it is facilitated by denervation of the target region (which can be achieved by neurochemical or mechanical forces), by application of a polypeptide growth factor (*e.g.*, TGF β , which increases the regional expression of cell adhesion molecules such as fibronectin and laminin), and by contacting the cells along a desired migratory path with a compound that inhibits a naturally occurring signal that would otherwise inhibit migration (*i.e.*, creating a permissive microenvironment by inhibiting an inhibitor).

Moreover, the shape of the migratory ridge can be controlled by varying the location of the infusion (*e.g.*, by altering the placement of the infusion cannula or of a biodegradable capsule containing the active compound(s) of the invention). Similarly, the number of cells within the ridge can be controlled by varying the dosage of the active compound(s) (*e.g.*, the dosage of TGF α) or the distance at which it is released relative to the population of neural precursor cells in the subependymal region. As described below (and as illustrated in Figs. 10A, 10B, 11A, 11B, and 11C), when animals received mid-striatal or medial striatal infusions, the migrating cells stopped before they reached the infusion cannulae, which resulted in S-shaped or L-shaped ridges. Thus, it is possible not only to facilitate the cells' migration, but to control where that migration ends. Adjusting the dose and the location of release of the growth factor (and perhaps other compounds) may allow restriction of the area affected to a relatively limited target region.

The proliferation and migration of neural precursor cells in the adult mammalian brain are distinct events that can be controlled separately.

Intracerebroventricular (ICV) or intrastriatal infusions of TGF α or EGF without deafferentation can induce proliferation, but degenerating, damaged (*e.g.*, by deafferentation or other injury), or otherwise abnormal (*i.e.*, malfunctioning) cells must be present to facilitate migration, at least on a scale that is large enough to impact recovery from an associated neurological deficit. As described further below, one can mimic the facilitating effect of degenerating, damaged, or otherwise abnormal cells with pharmacological agents. For instance, one can stimulate transient expression of cell adhesion molecules in the striatum by administering an inductive compound. For example, fibronectin is strongly upregulated by transforming growth factor beta (TGF β) in cultures of cerebellar astrocytes (Baghdassarian *et al.*, *Glia* 7:193-202, 1993). In transgenic mice overexpressing TGF β , fibronectin and laminin are also strongly increased in the CNS over normal levels (Wyss-Coray *et al.*, *Am. J. Pathol.* 147:53-67, 1995). Thus, directing migration with any compound that stimulates the expression of extracellular matrix molecules or cell adhesion molecules, particularly along the desired path of migration, is considered within the scope of the present invention.

Forebrain neural stem cells, which give rise to the migrating progenitors, are believed to remain in place along the ventricular wall (Morshead *et al.*, *Neuron* 13:1071-1082, 1994). In the experiments described below, a region of intense EGF receptor hybridization persisted along the lateral ventricle after the migratory ridge had moved into the striatum. In addition, elongated cells were always found between the ridge and the lateral ventricle. Thus, despite the mass cellular migration away from the subependymal zone, the stem cells themselves likely were not part of the migrating ridge. These neural stem cells would provide a renewable source of new neurons and glia. Therefore, multiple waves of neural progenitor cells can be stimulated to migrate into regions of the brain that are injured, or that have degenerated or that otherwise contribute to a neurological deficit. The persistence of these cells also suggests that the normal function of stem cells in the adult forebrain -- presently thought to provide new neurons for the olfactory bulbs -- should not be irreversibly disrupted by the treatments.

Abundant striatal expression of TGF α (and its mRNA) and a lack of dopaminergic innervation also characterize the early developing striatum (Weickert and Blum, *Devel. Brain Res.* 86:203-216, 1995; Bayer, *Intl. J. Devel. Neurosci.* 2:163-175, 1984). Similarly, the increased EGF receptor mRNA expression in the subependymal region in TGF α -infused animals mimics the abundant EGF receptor mRNA hybridization observed in the periventricular neuroepithelium in the developing brain (Seroogy *et al.*, *Neuroreport* 6:105-108, 1994; Seroogy *et al.*, *Brain Res.* 670:157-164, 1995). Messenger RNAs encoding forms of fibronectin, and its receptor, and other cell adhesion molecules, which may facilitate the migration of neural precursors, are also developmentally regulated (Pesheva *et al.*, *J. Neurosci. Res.* 20:420-430, 1988; Prieto *et al.*, *J. Cell Biol.* 111:685-698, 1990; Pagani *et al.*, *J. Cell Biol.* 113:1223-1229, 1991; Linnemann *et al.*, *Int. J. Devel. Neurosci.* 11:71-81, 1993). Thus, one way to conceptualize the effects observed in the TGF α -infused and 6-OHDA lesioned animals in the present studies is as a selective recapitulation of embryonic neurogenesis. That is, neural stem cells in the adult mammalian brain may respond to proliferation signals and their progeny may respond to migration signals as they do in the developing animal.

Neural stem cells have recently been found in subependyma throughout the adult rodent CNS (Weiss, *Soc. Neurosci. Abstr.* 25:101, 1995; Ray *et al.*, *Soc. Neurosci. Abstr.* 22:394.5, 1996) and in the subependyma of the adult human forebrain (Kirschenbaum *et al.*, *Cerebral Cortex* 4:576-589, 1994). According to the methods described herein, these cells can be manipulated to provide a source of new neurons for diseased, injured, or otherwise damaged or malfunctioning CNS neurons in diverse regions of the brain and spinal cord.

A. Advantages of the Present Invention

As described further below, one of the techniques proposed for treating a neurological deficit involves removing neural precursors from a patient who has such a deficit and growing those cells in culture to generate large numbers of neural progenitors. The cells may then be re-implanted into the same patient using techniques known to those of ordinary skill in the art (*e.g.*, see Stein *et al.*, In: Brain Repair, pp. 87-103, Oxford University Press, New York, 1995, or Leavitt *et al.*, *Soc. Neurosci. Abstr.* 22:505, 1996). Clearly, this technique is advantageous to those presently in use that require embryonic cells from aborted

fetuses; it avoids altogether the ethical issues raised by the need to use aborted fetuses as tissue donors. In addition, it is more likely to succeed because it will not stimulate the immune response that is responsible for a high incidence of transplant rejection.

Stimulating proliferation and migration of neural precursors *in vivo* has additional advantages; *in vivo* stimulation reduces the extent and possibly the number of invasive neurosurgical procedures. No stem cell excision surgery would be performed and multiple plugs of transplanted cells, which are typically required with embryonic or cultured cell grafts, would not be necessary. Further, there would be no massive die-off of undifferentiated neural progenitor cells due to the transplantation procedure. Typically, with human fetal dopaminergic cell grafts, 90% to over 99% of the implanted cells die before they become established in the host brain (Freed *et al.*, *Soc. Neurosci. Abstr.* 22:481.3, 1996).

Another advantage provided by the present invention is that neural progenitor cells would not be isolated from the host brain by scar tissue. Plugs of transplanted cells become encapsulated within an envelope of glyotic scar tissue and reactive astrocytes. In addition to the physical barrier of the dense glyotic tissue, reactive astrocytes within the scar tissue release factors that inhibit neurite outgrowth (McKeon *et al.*, 1995). Neural progenitors created *in vivo* are not isolated from the rest of the brain by scar tissue. The outgrowth of their neurites, therefore, would not be inhibited by a massive proliferation of reactive astrocytes. The directed migration provided for herein therefore allows selective repopulation (which may vary in extent) of specific injured regions of the CNS with large numbers of new cells without disturbing undamaged areas.

The techniques presented here also represent an advance over the single previous study of forebrain neural stem cells stimulated *in vivo*. In that study, adult rats received ICV infusions of EGF for six days and were followed for up to seven weeks post-infusion (Craig *et al.*, *J. Neurosci.* 16:2649-2658, 1996). In the present study, TGF α was infused for fourteen days and followed for up to three months following the end of infusion. This difference is critical in that only in the present study did the cells of the periventricular expansion migrate *en masse* into the overlying striatum. The *directed* mass migration of neural progenitors into a selected target area represents a much preferred method to repopulate degenerated brain regions with new neurons.

One area of intense recent interest is the manipulation of neural stem cell differentiation. Both the final location and the neurochemical phenotypes of the cells once they have differentiated are of primary importance and are discussed further below.

When neural precursor cells were removed from adult rodent brains and differentiated *in vitro*, cells immunochemically identified as astrocytes, oligodendrocytes and neurons are seen (Reynolds and Weiss, *Science* 255:1707-1710, 1992; Reynolds *et al.*, *J. Neurosci.* 12:4565-4574, 1992; Lois and Alvarez-Buylla, *Proc. Natl. Acad. Sci. USA* 90:2074-2077, 1993). Many of the cells identified as neurons were also immunoreactive for GABA and substance P, neurochemical markers for two cell types normally found in the striatum. Precursor cells explanted from the adult human brain also expressed neuronal markers and displayed electrophysiological properties associated with neurons (Kirschenbaum *et al.*, *Cerebral Cortex* 4:576-589, 1994).

These experiments suggest that when cells of the striatal ridge spontaneously differentiate *in vivo*, many of them will become cells with phenotypes typical of striatal neurons. Some recent data suggests that their phenotypes can be altered by exposure to different combinations of neurotrophins (Lachyankar *et al.*, *Soc. Neurosci. Abstr.* 22:394.7, 1996). Progenitor cells receiving different treatments expressed different neurochemical immunomarkers once they differentiated, including acetylcholinesterase, GABA, tyrosine hydroxylase (TH), and calbindin. The expression of TH was particularly interesting, since combined proliferation, migration and directed differentiation into dopamine cells could provide a novel method to replace striatal dopamine lost in Parkinson's disease (PD).

In PD patients, functionally significant numbers of new dopamine-producing striatal cells would aid in the reversal of motor deficits in a manner similar to transplants of aborted fetal midbrain tissue. In patients with Huntington's disease (HD), neural precursors would be stimulated to repopulate the striatum with new medium spiny GABAergic and other neurons lost to the disease. Some recent evidence from a different line of research indicates that reconstruction of the striatopallidal pathway itself might be possible. Conditionally immortalized neural progenitor cells transplanted into the striatum differentiated and sent processes from the striatum to the globus pallidus (Lundberg *et al.*, 1996).

B. Neurological Deficits Amenable to Treatment

Because the invention rests on the discovery that multipotent precursor cells can be stimulated to divide and migrate through the brain, it can be used to treat neurological deficits caused by a wide variety of diseases, disorders, and injuries. These insults include, but are not limited to, the following (others of skill in the art may categorize differently the diseases and disorders listed below; however categorized, the neurological deficits with which they are associated are amenable to treatment according to the methods of the present invention).

1. Degenerative Diseases

Degenerative diseases that can be treated according to the methods of the invention include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Pick's disease, progressive supranuclear palsy (PSP), striatonigral degeneration, cortico-basal degeneration, childhood disintegrative disorder, olivopontocerebellar atrophy (OPCA; including a heritable form), Leigh's disease, infantile necrotizing encephalomyelopathy, Hunter's disease, mucopolysaccharidosis, various leukodystrophies (such as Krabbe's disease, Pelizaeus-Merzbacher disease, and the like), amaurotic (familial) idiocy, Kuf's disease, Spielmayer-Vogt disease, Tay Sachs disease, Batten disease, Jansky-Bielschowsky disease, Reye's disease, cerebral ataxia, chronic alcoholism, beriberi, Hallervorden-Spatz syndrome, cerebellar degeneration, and the like.

2. Traumatic and Neurotoxic Injuries to the Central Nervous System

Traumatic and neurotoxic injuries that can be treated according to the methods of the invention include gunshot wounds, injuries caused by blunt force, injuries caused by penetration injuries (*e.g.*, stab wounds), injuries caused in the course of a surgical procedure (*e.g.*, to remove a tumor or abscess from the CNS or to treat epilepsy), poisoning (*e.g.*, with MPTP or carbon monoxide), shaken-baby syndrome, adverse reactions to medication (including idiosyncratic reactions), drug overdose (*e.g.*, from amphetamines), post-traumatic encephalopathy, and the like.

3. Ischemia

Any disruption of blood flow or oxygen delivery to the nervous system can injure or kill cells, including neurons and glial cells, therein. These injuries can be treated according to the methods of the present invention and include injuries caused by a stroke (including a global stroke (as may result from cardiac arrest, arrhythmia, or myocardial

infarction) or a focal stroke (as may result from a thrombus, embolus, hemorrhage, or other arterial blockage)), anoxia, hypoxia, partial drowning, myoclonus, severe smoke inhalation, dystonias (including heritable dystonias), acquired hydrocephalus, and the like.

4. Developmental Disorders

Developmental disorders that can be treated according to the methods of the invention include schizophrenia, certain forms of severe mental retardation, cerebral palsy (whether caused by infection, anoxia, premature birth, blood type incompatibility: etc. and whether manifest as blindness, deafness, retardation, motor skill deficit, etc.), congenital hydrocephalus, metabolic disorders affecting the CNS, severe autism, Down Syndrome, LHRH/hypothalamic disorder, spina bifida, and the like.

5. Disorders Affecting Vision

Disorders affecting vision, particularly those caused by the loss or failure of retinal cells, can be treated according to the methods of the invention. These disorders include diabetic retinopathy, serious retinal detachment, retinal damage associated with glaucoma, traumatic injury to the retina, retinal vascular occlusion, macular degeneration, heritable retinal dystrophies, optic nerve atrophy, and other retinal degenerative diseases.

6. Injuries and Diseases of the Spinal Cord

Injuries to or diseases affecting the spinal cord can also be treated according to the methods of the invention. Such injuries or diseases include post-polio syndrome, amyotrophic lateral sclerosis, nonspecified spinal degeneration, traumatic injury (such as those caused by automobile or sporting accidents), including any injury that crushes, partially severs, completely severs, or otherwise adversely affects the function of cells in the spinal cord), injuries caused by surgery to the spinal cord (*e.g.*, to remove a tumor), anterior horn cell disease, paralytic diseases, and the like.

7. Demyelinating or Autoimmune Disorders

Neurological deficits caused by demyelination or an autoimmune response can be treated according to the methods of the invention. Such deficits can be caused by multiple sclerosis, possibly lupus, and others.

8. Infectious or Inflammatory Diseases

Neurological deficits caused by an infection or inflammatory disease can be treated according to the methods of the invention. Infections or inflammatory diseases that

can cause treatable deficits include Creutzfeldt-Jacob disease and other slow virus infectious diseases, AIDS encephalopathy, post-encephalitic Parkinsonism, viral encephalitis, bacterial meningitis and meningitis caused by other organisms, phlebitis and thrombophlebitis of intracranial venous sinuses, syphilitic Parkinsonism, tuberculosis of the CNS, and the like.

5 9. Miscellaneous

Those of ordinary skill in the art are well able to recognize neurological deficits, regardless of their cause, and to apply the methods of the present invention to treat patients who have such deficits. In addition to the conditions listed above, which are amenable to treatment with the methods described herein, neurological deficits can be caused
10 by Lesch-Nyhan syndrome, myasthenia gravis, various dementias, numerous parasitic diseases, epilepsy, and the like. The methods of the invention can be readily applied to alleviate neurological deficits caused by these and other diseases, disorders, or injuries.

C. Polypeptides that bind the EGF Receptor

1. The EGF Family

15 Polypeptides in the EGF family appear, in some ways, unrelated. For example, TGF α and EGF have only 30% structural homology (Marquardt *et al.*, *Science* 223:1079-1082, 1984). However, they display similar binding kinetics for, and stimulate tyrosine-specific phosphorylation of, the M_r 180,000 EGF membrane receptor (Cohen *et al.*, *J. Biol. Chem.* 255:4834-4842, 1980; Reynolds *et al.*, *Nature* 292:259-262, 1981). The
20 functional equivalence of the two growth factors is partly attributed to the same relative positioning of six cysteine residues, represented by "C" in the consensus sequence: CX₇CX_{4,5}CX₁₀CXCX₈C. These conserved residues impose similar disulfide bond-mediated structural constraints and, thus, a related three-dimensional structure (Twardzik *et al.*, *Proc. Natl. Acad. Sci. USA* 82:5300-5304, 1985). Those of ordinary skill in the art are well able to
25 compare any given amino acid sequence with the EGF-family consensus sequence to determine whether a polypeptide is likely to be functionally equivalent to EGF (and, if so, useful in practicing the methods of the present invention). (*see, e.g.*, Blomquist *et al.*, *Proc. Natl. Acad. Sci. USA* 81:7363-7367, 1984, for a description of a computer search that revealed a similar pattern of cysteine and glycine residues in EGF, TGF α , and the sequence
30 of a 19 kDa early protein of vaccinia virus).

In addition to EGF, TGF α , and vaccinia growth factor (VGF), the EGF family is known to include amphiregulin (AR), betacellulin (BTC), epiregulin (ER), heparin-binding EGF-like growth factor (HB-EGF), schwannoma-derived growth factor (SDGF), HUS 19878, myxomavirus growth factor Shope fibroma virus growth factor, and teratocarcinoma-derived growth factor-1 (TDGF-1; also known as Cripto-1 (CR-1)).

2. Methods for Determining EGF Receptor binding

Those of ordinary skill in the art are readily able to determine whether any given polypeptide binds the EGF receptor. As used herein, the term "binds" refers to any specific interaction between a polypeptide and the EGF receptor that results in signal transduction sufficient to elicit a biological response, preferably a response that contributes to the reduction of a neurological deficit. Preferably, any given polypeptide useful in the methods of the present invention will bind the EGF receptor with an affinity that is equivalent to at least 50%, more preferably at least 70%, and most preferably at least 90% of the binding affinity of EGF itself (see Twardzik *et al.*, *supra*, for a comparison of the biological activity of VGF, TGF α , and EGF in EGF receptor binding).

If guidance is required in performing an EGF receptor binding assay, those of skill in the art can consult any one of numerous publications describing a suitable procedure (the five publications on this topic that follow are hereby incorporated by reference in their entirety). For example, one could consult Cohen and Carpenter, *Proc. Natl. Acad. Sci. USA* 72:1317-1321, 1975) or, for a modification thereof, Twardzik *et al.*, *supra*. Similarly, for review of the EGF receptor, including specific binding and sequence information, signaling, and receptor topology, one may consult, for example, McInnes and Sykes (*Biopolymers* 43:339-366, 1997) Boonstra *et al.*, (*Cell Biol. Intl.* 19:413-430, 1995), or Gill (*Mol. Reprod. Dev.* 27:46-53, 1990).

D. Directed Migration

The examples below also provide evidence for successful directed migration of neural precursor cells, particularly in the adult rodent forebrain. The immuno-histochemical and other techniques employed in the working examples below (and described at length therein), as well as comparable techniques routinely performed by those of ordinary skill in the art, can be used to characterize the effect of any infusion of growth factor or any other stimulus applied to direct cellular migration. Indeed, it is possible to trace the cells'

migration in some detail (*i.e.*, the number of cells, their size, shape, and position within the nervous system can be determined).

A variety of stimuli can be applied to cells *in vivo* to direct their migration *en masse* (the term "en masse," when used herein to describe cellular migration, refers to the movement of a population of cells in substantially the same direction for a sufficient period of time to be visualized as a mass (as, *e.g.*, is apparent in Figs. 9, 10, and 11)). Broadly, one can direct migration in one of two ways: (1) in a conducive manner, *i.e.*, by applying a stimulus that positively attracts migrating cells (such as a chemoattractant, neurotropic factor, or a compound (*e.g.*, TGF β) that increases the expression of a cell adhesion molecule or extracellular matrix molecule, *e.g.*, fibronectin, laminin, or a neural cell adhesion molecule), or (2) in a permissive manner, *i.e.*, by applying a stimulus that inhibits a signal that would otherwise inhibit migrating cells.

The stimuli that direct migration include disruption of the tissue in the target area (which may be the site where cells have been damaged, *e.g.*, the striatum or substantia nigra, where dopaminergic cells are known to be lost in association with a number of debilitating neurodegenerative diseases; the cerebral cortex, where neurons and glia are lost following an ischemic episode caused by, *e.g.*, a thrombus or embolus; or the spinal cord, where motor neurons are lost due to, *e.g.*, a traumatic injury; or may be any site where cells make abnormal connections due to a developmental disorder). Alternatively, tissue may be disrupted in or along a path extending from the source of the neural progenitor cells to the desired endpoint of their migration.

The tissue may be disrupted by physical force (*e.g.*, ablating or excising neurons, or severing one or more of the processes that extend from the neuronal cell bodies) or by applying a chemical substance such as a toxin or neurotoxin (*e.g.*, ricin or 6-OHDA), a corrosive chemical (*e.g.*, an acidic or basic solution), a compound that induces apoptosis (*see, e.g.*, Leavitt *et al.*, *Soc. Neurosci. Abstr.* 22:505, 1996), a compound that induces demyelination (*see, e.g.*, Lachapelle *et al.*, *Soc. Neurosci. Abstr.* 23:1689, 1997), or a compound capable of inhibiting the activity of the cell, *e.g.*, an antisense oligonucleotide (such as an oligonucleotide that inhibits transcription of the gene encoding the cell's primary neurotransmitter), an antibody, or a polypeptide. Many such compounds are known to those of ordinary skill in the art and include compounds that bind to, but fail to activate, a receptor

on the cell surface, such as the metabotropic receptors normally bound by glutamate. For example, in the studies described below, the effect of dopamine denervation with 6-OHDA (together with infusion of TGF α) on cellular migration is apparent.

Those of ordinary skill in the art are well able to direct cellular migration by applying any of the chemical substances described above to a targeted area of the nervous system, particularly given the remarkably clear and accurate images of a patient's brain and spinal cord that can now be generated with, *e.g.*, magnetic resonance imaging or computed tomographic scans.

Moreover, it is apparent from the studies described below that altering one or more of the variables associated with application of a compound that directs migration (those variables including the nature, position, concentration, and duration of the application) can be altered to direct more precisely the migratory path the cells follow and to define the place at which they come to rest.

E. Differentiation Factors

While some neural precursor cells may spontaneously differentiate, given enough time, substantially greater benefit can be realized by controlling when and where differentiation takes place; exerting such control allows one to limit neural "repopulation" (which may be partial or complete, so long as it is sufficient to reduce a neurological deficit) to areas of the CNS in need thereof. Accordingly, various stimuli can be administered before, during, or after contacting neural progenitor cells with a polypeptide that binds the EGF receptor.

Broadly, the stimuli inducing differentiation can be "general" or "directed." A general differentiation stimulus is one that stimulates a cell to differentiate as it naturally would and is applied whenever a neurological deficit can be reduced by stimulating a cell to express its natural phenotype. For example, it is known in the art that cells from the striatal subependymal zone differentiate into GABAergic neurons upon exposure to general differentiation signals. Thus, stimulating these cells to differentiate by applying a general differentiation factor would reduce the neurological deficits associated with Huntington's Disease; in these patients, GABAergic medium spiny neurons are lost selectively.

Those of ordinary skill in the art are well able to apply the known means of stimulating general differentiation to stimulate differentiation of the proliferating and

migrating cells of the present invention. For example, contacting cells with a retinoblastoma protein is known to cause them to exit the cell cycle, a requirement for differentiation (for a recent study, *see*, Slack *et al.*, *J. Cell Biol.* 140:1497-1509, 1998) and contacting cells with a cell cycle associated kinase inhibitor, p21, can maintain cells in a post-mitotic (*i.e.*,
5 differentiated) state (Berger *et al.*, *Soc. Neurosci. Abstr.* 22:505, 1996). Another stimulus that can be applied to stimulate differentiation in the context of the present methods is a cyclin D cell cycle regulator (Ouaghi *et al.*, *Soc. Neurosci. Abstr.* 22:1706, 1996). Should one wish to stimulate differentiation of oligodendrocyte precursors, integrins may be applied (*see, e.g.*, Buttery *et al.*, *Soc. Neurosci. Abstr.* 22:1723, 1996). Brain-derived neurotrophic
10 factor (BDNF) and retinoic acid (RA) are well known for their abilities to stimulate cellular differentiation (*see, e.g.*, Ahmed *et al.*, *J. Neurosci.* 15:5765-5778, 1995).

In the event neural precursor cells are cultured prior to directing migration, they may be transplanted into an area of the brain that is capable of influencing their differentiation. For example, a higher percentage of transplanted neural precursor cells
15 differentiated into neurons when placed near the subventricular zone (up to 35%) compared to those transplanted to more lateral sites (where only 0-8% of the cells differentiated) (Catapano and Macklis, *Soc. Neurosci. Abstr.* 23:345, 1997). Similarly, transplanted cerebellar precursors express markers for hippocampal neurons when they are transplanted into the hippocampus (Vicario-Abejon *et al.*, *J. Neurosci.* 15:6351-6363, 1995).

Accordingly, one of ordinary skill in the art will appreciate that, as an alternative to
20 contacting the progeny of proliferating neural progenitor cells with a compound that stimulates their differentiation, the invention can be practiced by transplanting cells in or sufficiently near a region of the brain that is capable of directing their differentiation.

A directed differentiation stimulus is one that stimulates a cell to differentiate,
25 but with a phenotype that is different from the one it would naturally express. A directed differentiation factor would be applied whenever a neurological deficit could be reduced by stimulating a cell to express a non-natural phenotype. One such instance is in the case of Parkinson's disease, where differentiation of striatal cells into dopamine-producing cells could substitute for the loss of dopaminergic innervation from the substantia nigra.

Similarly, one would aim to stimulate expression of a cholinergic phenotype in the septal
30 region, where cells are selectively lost in Alzheimer's Disease; in spinal motor neurons,

which are lost in amyotrophic lateral sclerosis and following traumatic spinal injuries; and in oligodendrocytes, which are lost in demyelination disorders such as multiple sclerosis.

Factors from the GDNF/neurturin (TGF β) family, which are derived from a glial cell line, may induce differentiation in neural cells (which is, moreover, enhanced by RA) (Hishiki *et al.*, *Cancer Res.* 58:2158-2165), and GDNF stimulates motor neuron differentiation in rat ventral mesencephalic cultures. BDNF and ciliary neurotrophic factor (CNTF) also promote motor neuron differentiation (their effects appear to be additive or synergistic to the effects of GDNF) (Zurn *et al.*, *J. Neurosci. Res.* 44:133-141, 1996). In addition, motor neuron differentiation can be induced by application of vitronectin, which is expressed in the ventral region of the neural tube (Martinez-Morales *et al.*, *Development* 124:5139-5147) and by the protein encoded by sonic hedgehog (Tanabe *et al.*, *Curr. Biol.* 5:651-658, 1995). A member of the sonic hedgehog family, Indian hedgehog, is expressed in developing and mature retina and promotes retinal progenitor proliferation and photoreceptor development (Levine *et al.*, *J. Neurosci.* 17:6277-6288, 1997).

Cortical neural progenitors adopt a region-specific phenotype influenced by EGF, TGF α and the type of substrate upon which they are grown. EGF or TGF α doubled the percentage of limbic neurons derived from non-limbic- area precursors when they were plated on growth factor-deficient Matrigel or collagen type IV (Ferri *et al.*, *Development* 121:1151-1160, 1995).

Insulin is known to affect differentiation of fetal neuron cell cultures, even more than IGF-1 (Abboud *et al.*, *Soc. Neurosci. Abstr.* 23:1425, 1997). Basic fibroblast growth factor (bFGF) and neurotrophins can be used to direct the differentiation of hippocampal cells (Vicario-Abejon *et al.*, *Neuron* 15:105-114, 1995).

In one embodiment, the methods of the invention can be applied to restore neural pathways that are lost to degenerative illness. For example, differentiated striatal GABAergic neurons can restore striatopallidal projections upon their differentiation. Those of ordinary skill in the art are well able to recognize numerous neural pathways that are amenable to reconstruction by the methods of the present invention.

F. Pharmaceutical Compositions

Polypeptides suitable for use in the present invention (*i.e.*, those that bind the EGF receptor or stimulate differentiation, also referred to herein as "active compounds") can

be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise one or more polypeptides and a "pharmaceutically acceptable carrier," a term which is intended to include any and all solvents, dispersion media, coatings, antibacterial agents, antifungal agents, isotonic and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated.

Those of ordinary skill in the art appreciate the need to formulate pharmaceutical compositions for their intended route of administration (which may include parenteral, *e.g.*, intravenous, intradermal, or intramuscular injection; oral administration; or direct application to the affected area). It is contemplated that the present methods will be carried out by applying polypeptides to neural precursors harvested from the brain and placed in culture or directly to the precursor cells *in vivo* (by, *e.g.*, infusion through an injection cannula or shunt, or by implantation within a carrier, *e.g.*, a biodegradable capsule) but other routes of administration, particularly parenteral (preferably intravenous) administration, are also within the scope of the invention.

Solutions or suspensions useful in the pharmaceutical compositions of the present invention (*e.g.*, in a composition containing a polypeptide that binds the EGF receptor and a compound that stimulates the differentiation of neural precursors) can include: sterile diluents such as water, normal saline, fixed oils, polyethylene glycols, glycerine, propylene glycol, or other synthetic solvents; antibacterial or antifungal agents such as benzyl alcohol, parabens (*e.g.*, methyl parabens), chlorobutanol, phenol, ascorbic acid, thimerosal, and the like; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates, or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where the active compound is water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor

EL™ (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists (proper fluidity can be maintained, for example, by using coatings such as lecithin, by maintaining a certain particle size in the case of dispersion, and by including surfactants).

5 The composition must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi, as described above. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition.

10 Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

15 Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a polypeptide that binds the EGF receptor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of the active compound plus any
20 additional desired ingredient from a previously sterile-filtered solution.

25 In one embodiment, the active compound is prepared with one or more carriers that will protect it against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparing such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially, for example, from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in
30 the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate the compositions of the invention in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound, the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

In lieu of direct application of polypeptides that bind the EGF receptor or stimulate cellular differentiation, nucleic acid molecules encoding those polypeptides can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Patent 5,328,470) or by stereotactic injection (*see, e.g., Chen et al., Proc. Natl. Acad. Sci. USA* 91:3054-3057, 1994). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded, for example, in the brain or spinal cord. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.,* retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

G. Treatment Regimes

By way of example (those of ordinary skill in the art are well able to extrapolate from one model (be it an *in vitro* or *in vivo* model) to another, progressing toward optimal dosages for human patients), for a rodent brain, the infusions to stimulate proliferation of neural precursor cells were continued for a period of at least two weeks. The result being a dramatic increase in the numbers of undifferentiated progenitor cells along the adjacent ventricle. The continuous TGF α infusion applied in the working examples below also supports radial cellular migration, but is not sufficient, by itself, to stimulate the massive radial migration observed in certain animals. The duration of any treatment performed

according to the methods described herein can be varied according to the desired results. For example, in the working examples below, the cells greatly increased their numbers for more than a week prior to their mass migration away from the ventricle. Moreover, delayed migration was facilitated by denervation of the target region, either neurochemically or mechanically, and may also be facilitated pharmacologically by the concurrent infusion of factor(s) such as another neurotrophic factor, TGF β , which increases the regional expression of cell adhesion molecules believed to underlie the radial migration (as described above). The pattern of migration and the final location of the ridge of migrating cells can be controlled by altering the location of the infusion.

Those of ordinary skill in the art are well able to determine the required dosage of a compound administered in the context of the present invention. Preferably, the dosage will range, whether infused or released from a time-release vesicle, from 1 to 100 ng/kg/day of the active compound or ingredient (*e.g.*, TGF α or any of the factors described above); more preferably, 1 to 50 ng/kg/day; and most preferably, 1 to 10 ng/kg/day will be administered.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Expression of TGF α and EGF Receptor mRNAs in the Normal Developing and Adult Nigrostriatal System

The following example describes studies that demonstrate that TGF α and EGF receptor mRNAs in the normal developing and adult nigrostriatal system.

As reviewed in the Detailed Description, mRNAs encoding EGF-family neurotrophic factors are developmentally regulated in the nigrostriatal system. In the studies described below, the expression of TGF α and EGF receptor mRNAs is examined in the normal developing and adult rodent system.

A. Animals and Tissue Preparation

Adult male adult and timed-pregnant female Sprague-Dawley rats (250-350 g) were obtained from Simonsen (Gilroy, CA). For these experiments and all others described herein, the animals were maintained in a temperature and humidity controlled vivarium. Use of the animals for all of the experimental procedures employed was approved by the University of California, Irvine, Animal Research Committee in accordance with National Institutes of Health guidelines.

Newborn (P0), postnatal day 1 (P1), and P4 animals were anesthetized by hypothermia and sacrificed by decapitation. P10, P21, and adult animals were sacrificed by decapitation. Their brains were quickly removed, frozen in isopentane at -20°C, and stored at -70°C. Coronal cryostat sections were cut at 20 μ m and thaw-adhered to Vectabond™ (Vector Labs, Inc.) coated slides in ordered anterior-to-posterior rows. The sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hour, rinsed in phosphate buffer and air dried. Sections were stored with desiccant at -20°C until processed.

B. Hybridization Probes

TGF α mRNA probes were generated from a 550 nucleotide XbaI/BamHI cDNA fragment from the 5' end of rat TGF α and subcloned into pGEM 7Zf (Promega, Inc.). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor mRNA probes were produced from a 718 base pair BamHI/SphI insert from the 5' end of the gene, in pGEM 7Zf. Probes for rat TH were created using the 1.2 kb BamHI/EcoRI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of [³⁵S]UTP (NEN Research Products, Inc.).

C. In situ Hybridization and Analysis

In situ nucleic acid hybridization was performed according to the method described by Simmons *et al.*, (*J. Histotech.* 12:1169-181, 1989) except that developing brains were treated with 0.0001% proteinase K solution and 0.05 M EDTA. Sections were hybridized overnight at 65°C with sense or antisense probes at a concentration of 10⁷ cpm/ml. Adjacent sections from the same animals were hybridized to each of the probes so that direct comparisons could be made of their anatomical distributions.

Slides from developing and adult animals were grouped together and apposed with ^{14}C -labeled brain paste standards to autoradiographic BetaMax HyperfilmTM (Amersham, Inc.) for six to seven days. After successful development of the autoradiography film, the slides were dipped in Kodak NTB-2 emulsion and exposed for four weeks. The autoradiographic sheet film and NTB-2 emulsion were developed with D-19 developer and Rapid Fix (Kodak, Inc.). The brain sections were then counterstained with thionin and coverslipped. Dipped and stained sections were examined semiquantitatively and photographed under bright and dark field microscopy.

Expression of $\text{TGF}\alpha$ and EGF receptor mRNAs in the nigrostriatal system was traced through selected time points from early postnatal development to adulthood. $\text{TGF}\alpha$ mRNA hybridization was found in abundance in the early postnatal striatum but was gradually reduced to near adult levels by P21. Expression in the corpus callosum increased through postnatal development to levels comparable to those in the striatum. $\text{TGF}\alpha$ mRNA was not detected in significant abundance in the developing or the adult substantia nigra.

Striatal EGF receptor mRNA peaked early in postnatal development and decreased again by P21. EGF receptor was highest in the neuroepithelia around the lateral ventricles, but was also found at moderate levels in the body of the striatum. In the developing ventral midbrain, EGF receptor mRNA was barely detectable in early postnatal brains, but gradually increased to moderate levels by P21.

In adult animals, $\text{TGF}\alpha$ mRNA expression was moderate in the striatum and low-to-moderate in the ventral striatum and nucleus accumbens. EGF receptor mRNA hybridization was found at low levels in the body of the striatum and nucleus accumbens with higher punctate expression dispersed throughout. It persisted at moderate levels in the regions of striatum immediately bordering the lateral ventricles.

In the adult ventral midbrain, EGF receptor mRNA hybridization was found in the substantia nigra (SN), particularly the medial pars compacta, and the paranigral and parabrachial nuclei of the ventral tegmental area (VTA).

Previous studies indicated that $\text{TGF}\alpha$ and EGF receptor mRNAs are strongly regulated during ontogeny of the nigrostriatal system and that their expression in the adult largely represents a continuation of the developmental pattern (Lazar and Blum, *J. Neurosci.* 12:1688-1697, 1992; Weickert and Blum, *Devel. Brain Res.* 86:203-216, 1995). $\text{TGF}\alpha$ and

EGF receptor mRNA hybridization in the developing and adult animals closely paralleled the findings of these earlier reports. The persistence of their expression in the adult striatum and midbrain is consistent with a supportive role in the mature nigrostriatal system.

The moderate EGF receptor mRNA expression in the adult subependymal regions along the forebrain lateral ventricles suggests a role in the maintenance or function of cells in this region as well (Seroogy *et al.*, *Brain Res.* 670:157-164, 1995; Weickert and Blum, 1995, *supra*). TGF α (or EGF) has been shown to support the survival and differentiation of "EGF-responsive" cells from this region when they are explanted and grown *in vitro* (Reynolds and Weiss, *Science* 255:1707-1710, 1992; Reynolds *et al.*, *J. Neurosci.* 12:4565-4574, 1992). It may perform a similar function *in vivo* during development.

Example 2: Modulation of TGF α and EGF Receptor mRNA Expression by 6-hydroxydopamine Lesion and Striatal TGF α Infusion

The following example describes studies that demonstrate that methods and compositions of the invention can be used to modulate TGF α and EGF receptor mRNA expression. These studies also demonstrate that EGF infusions in lesioned animals induced cellular expansion and TGF α induced both cellular expansion and striatal ridge formation.

In situ hybridization was used to determine whether nigrostriatal TGF α or EGF receptor mRNA was altered by intrastriatal infusion of TGF α . In addition, the influence of unilateral 6-OHDA lesions on receptor expression in infused and uninfused animals was examined.

A. Treatment Groups

Adult male Sprague-Dawley rats weighing 250-300 grams were obtained from Simonsen (Gilroy, CA) and assigned to one of five treatment groups: (1) striatal TGF α infusion, nigral 6-OHDA lesion (hereafter, "lesion"); (2) TGF α infusion, no lesion; (3) artificial cerebrospinal (aCSF) infusion, lesion; (4) aCSF infusion, no lesion; (5) no infusion, no lesion. Four to eight animals were used per experimental group. The animals were monitored after each surgical procedure until fully recovered and maintained at all other times in a temperature and humidity controlled vivarium.

B. 6-hydroxydopamine Lesions

Rats were anesthetized with 8 mg xylazine and 100 mg ketamine per kilogram body weight. A chilled solution of 4.8 mg/ml 6-hydroxydopamine HCl (6-OHDA; Sigma Chemical Co.) in 0.9% saline with 0.01% ascorbic acid was prepared immediately before injection. Using sterile technique, an 8 μ l volume was stereotaxically injected into the left substantia nigra (+3.7 A/P; +2.1 M/L; +2.0 D/V) at a rate of 1 μ l/minute using interaural zero as a reference (Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, San Diego, 1986). The success and extent of 6-OHDA lesions were monitored by tyrosine hydroxylase (TH) mRNA *in situ* hybridization in the midbrain. TH is the rate-limiting enzyme in the dopamine synthetic pathway and is a common marker for dopamine-producing neurons. One animal with an incomplete lesion (retaining significant numbers of nigral TH-IR cells) was excluded from the study and is not included in the total number of animals.

C. Infusions

Osmotic minipumps (model 2002, Alzet, Inc.) were implanted four to five weeks post-lesion. The minipumps were filled with approximately 200 μ l of either 0.05 μ g/ml TGF α in artificial cerebrospinal fluid (aCSF) for experimental animals, or aCSF only for control animals, and incubated overnight at 37°C prior to implantation. Following anesthesia as above, and under sterile conditions, the 5 mm cannula attached to the minipump (brain infusion kit, Alzet, Inc.) was stereotaxically implanted into the left caudate-putamen (+1.2 A/P; +2.7 M/L) using Bregma as a reference (Paxinos and Watson, 1986, *supra*) and fixed to the skull with carboxylate cement (Fig. 1). The minipump itself was placed subcutaneously in the interscapular region. The infusate was delivered directly into the striatum over a period of two weeks at a rate of 0.5 μ l/hour.

D. Tissue preparation.

At the end of the infusion period, animals were sacrificed by decapitation. Their brains were quickly removed, frozen in isopentane at -20°C, and stored at -70°C. Coronal cryostat sections were cut at 20 µm and thaw-adhered to Vectabond™ (Vector Labs, Inc.) coated slides in ordered anterior-to-posterior rows. The sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour, rinsed in phosphate buffer and air dried. Sections were stored with desiccant at -20°C until processed.

E. Hybridization Probes.

TGFα mRNA probes were generated from a 550 nucleotide XbaI/BamHI cDNA fragment from the 5' end of rat TGFα subcloned into pGEM 7Zf (Promega, Inc.). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor mRNA probes were produced from a 718 base pair BamHI/SphI insert from the 5' end of the gene in pGEM 7Zf. Probes for rat TH were created using the 1.2 kb BamHI/EcoRI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of [³⁵S]UTP (NEN Research Products, Inc.).

F. In situ Hybridization.

In situ nucleic acid hybridization was performed according to the method described by Simmons *et al.* 1989, *supra*). Parallel sections from experimental and control animals were hybridized overnight at 65°C with sense or antisense probes at a concentration of 10⁷ cpm/ml. Adjacent sections from the same animals were hybridized to each of the probes so that a direct comparison could be made of their anatomical distributions.

Slides from experimental and control animals were grouped together and apposed with ¹⁴C-labeled brain paste standards to autoradiographic BetaMax Hyperfilm (Amersham, Inc.) for three to seven days. After successful development of the autoradiography film, the slides were dipped in Kodak NTB-2 emulsion and exposed for four weeks. The autoradiographic sheet film and NTB-2 emulsion were developed with D-19 developer and Rapid Fix (Kodak, Inc.). The brain sections were then counterstained with thionin and coverslips were applied.

G. Analysis and Quantitation

Dipped and stained sections were examined and photographed under bright and dark field microscopy. Autoradiograms were analyzed quantitatively using an MCID system (Microcomputer Imaging Device, Imaging Research, Inc.). Densitometry readings were sampled at multiple sites within each anatomical region of interest and averaged.

Relative concentrations of TGF α and EGF receptor hybridization were then estimated using a computer-generated third degree polynomial standard curve constructed from the ^{14}C brain paste standards. The estimated values for each region in each treatment group were then averaged and their standard errors calculated. Brain regions ipsilateral to the experimental treatments were compared to the corresponding contralateral regions in the same sections or to corresponding regions in control brains at approximately the same positions. Significance of the comparisons was determined using the Student's t-test.

H. Normal Expression and Control Infusions

Expression of TH, TGF α and EGF receptor mRNAs in the striatum, the striatal subependymal region, and the SN in control animals receiving striatal infusions of aCSF was indistinguishable from that in normal animals (See Example 1 for normal developing and adult expression). TH mRNA hybridization was prominent throughout the SN-VTA. TGF α mRNA was not detected in the SN. EGF receptor mRNA, however, was prominent in the medial substantia nigra pars compacta (SNc), the paranigral, parabrachial, and interpeduncular nuclei of the VTA (Fig. 2).

TGF α mRNA hybridization was expressed in the caudate-putamen and nucleus accumbens (NA), being slightly less dense in the NA. EGF receptor hybridization was found at low levels throughout the body of the striatum and NA with higher punctate expression dispersed throughout the low level background, and at moderate levels in the proliferative regions of striatum bordering the lateral ventricles.

I. Effects of 6-OHDA Lesions

Unilateral nigral 6-OHDA lesions reduced TGF α mRNA hybridization in the ipsilateral striatum by 25%, but had no effect on contralateral TGF α mRNA hybridization (Fig. 5). Striatal and subependymal EGF receptor hybridization were unchanged in lesioned animals compared to normal animals (Fig. 6). In the midbrain, 6-OHDA lesions abolished EGF receptor hybridization in the ipsilateral SN-VTA.

J. Effects of TGF α infusions.

In unlesioned animals receiving TGF α infusions, TGF α mRNA hybridization in the infused striatum was unchanged compared to the contralateral striatum or striata from normal animals (Fig. 5). A few of the animals receiving infusions of either TGF α or aCSF displayed a slight increase of TGF α mRNA hybridization immediately around the infusion cannula scar. TGF α mRNA hybridization in the substantia nigra was not increased to detectable levels by the infusions.

Hybridization to EGF receptor mRNA was dramatically increased in the ipsilateral subependymal region, but not in the rest of the striatum, in all animals receiving TGF α infusions (Fig. 6). No change from normal was observed in EGF receptor hybridization in the SNc with TGF α infusion alone.

K. Combined TGF α infusion and 6-OHDA lesion.

Striatal TGF α mRNA hybridization in animals receiving both striatal TGF α infusions and subsequent nigral 6-OHDA lesions was indistinguishable from that in lesion-only animals (Fig. 5). Similarly, EGF receptor hybridization in the midbrains of TGF α -infused/6-OHDA-lesioned animals was indistinguishable from that in lesion-only animals.

EGF receptor mRNA hybridization in the forebrain revealed an anomalous ridge of dense hybridization in the body of the ipsilateral striatum in addition to the increased hybridization in the subependymal region (Figs. 4, 6, and 7). The ridge was found in five of the six rats in the combined TGF α infusion/lesion group, but only in one of six rats in the TGF α infusion/nonlesion group. EGF receptor hybridization in the surrounding striatum was unchanged.

L. Discussion

The results described above permit analysis of the modulatory effects of nigral 6-OHDA lesions or striatal infusions of TGF α , or both, on the expression of mRNAs encoding TGF α and the EGF receptor in the adult rodent nigrostriatal system. The results clearly demonstrated changes in expression associated with each treatment individually and a unique pattern of striatal expression when the two treatments were combined.

1. Effects of 6-OHDA lesions.

Midbrain lesions with 6-OHDA reduced TGF α mRNA hybridization in the striatum by 25% at several weeks post-lesion. If TGF α peptide levels parallel expression of TGF α mRNA in this system, the decrease in TGF α mRNA may be one aspect of the rodent

lesion model that is not similar to idiopathic human PD: TGF α is greatly *increased* in the striata of PD patients (Mogi *et al.*, *Neurosci. Lett.* 180:147-150, 1994). TGF α has been shown to enhance a number of measure of dopamine neuron function *in vitro* (Alexi and Hefti, *Neurosci.* 55:903-918, 1993). The increase of TGF α (and EGF and other trophic factors) may therefore reflect a response to the continuing degeneration of dopamine neurons and their striatal efferents and may contribute to the capacity of remaining midbrain dopamine cells to compensate for the lost striatal dopaminergic innervation (Mogi *et al.*, 1994, *supra*). Thus, a partial- or perhaps a chronic-injury model might better represent this aspect of human PD.

The difference in the time course of the loss of dopamine cells may also help explain the apparent discrepancy between the 6-OHDA rodent model and human PD. In the rat model, midbrain dopamine neurons are killed relatively quickly by a single injection of neurotoxin. The chronic, progressive degeneration of mesencephalic dopamine neurons in human PD occurs over many years. In the present study, the animals were sacrificed well after midbrain dopamine cells had degenerated. There may have been early changes in striatal TGF α mRNA expression in these animals that would not be apparent in our experiments. It would be of interest to determine how TGF α mRNA expression varies in the rat model over shorter periods post-lesion, while dopamine neurons are in the process of degenerating.

Moderate TGF α mRNA expression in the caudate-putamen is consistent with its putative role as a target-derived growth factor for midbrain dopamine neurons. The underlying cause of its decrease in the ipsilateral striatum after midbrain neurotoxic lesion is unclear. Dopamine receptor binding has been shown to influence TGF α mRNA expression in the hypothalamus (Borgundvaag *et al.*, *Endocrinol.* 130:3453-3458, 1992), but no such interaction has yet been demonstrated in the striatum. TGF α mRNA is expressed in subpopulations of neurons and glia in the normal adult rodent striatum (Seroogy *et al.*, *J. Neurochem.* 60:1777-1782, 1993). Dopamine denervation of the striatum could potentially have influenced TGF α mRNA expression in postsynaptic neurons, astrocytes or both.

Contralateral striatal TGF α mRNA expression was not significantly altered by 6-OHDA lesion. This finding, too, is consistent with a dopamine denervation-mediated decrease in TGF α mRNA expression. Only a few percent of mesostriatal dopaminergic

projections are contralateral (Loughlin and Fallon, *Neurosci. Lett.* 32:11-16, 1982), thus any contralateral regulatory effects resulting from the lesion would be expected to be minor compared to ipsilateral effects.

EGF receptor mRNA hybridization in the DA-denervated striatum did not differ significantly from that in the contralateral CP or in unlesioned control striata. Again, there may have been early changes in expression due to the midbrain lesion or implantation of the infusion cannula that were not manifest at several weeks postlesion or two weeks post-implantation. The abolition of EGF receptor mRNA hybridization in the lesioned SNc confirms a similar observation after 6-OHDA lesion of the medial forebrain bundle (Seroogy *et al.*, *Neuroreport* 6:105-108, 1994). This lesion-induced decrease was previously cited as evidence of EGF receptor expression by nigral dopamine neurons (Seroogy *et al.*, 1994, *supra*), but the possibility remains of nigral glial production of EGF receptor mRNA that is subject to regulation by injury or death of nearby nigral dopamine neurons. Interestingly, EGF receptor binding in the midbrains of postmortem PD patients is unchanged from normals (Villares *et al.*, *Brain Res.* 628:72-76, 1993). Thus, the loss of EGF receptor mRNA expression after lesion represents another difference between the rodent lesion model and human PD. As with TGF α expression in the striatum, a partial-lesion model may better mimic in a rat brain the changes seen in a Parkinsonian human brain.

2. Effects of TGF α infusions.

In unlesioned animals, infusion of TGF α or aCSF did not significantly alter TGF α mRNA hybridization from normal levels in the midbrain or striatum. Despite reports of autostimulation of TGF α expression in other tissues or cell types (Coffey *et al.*, *Nature* 328:817-820, 1987; Barnard *et al.*, *J. Biol. Chem.* 269:22817-22822, 1994), results from the present study do not provide evidence for such activity in this system. The autostimulatory effects in those earlier studies were produced on the order of a few hours. Brain tissue in the present study was obtained after continuous exposure to the growth factor over a period of two weeks. Thus, an early upregulation near the beginning of the infusion that later subsided would not be evident.

As with TGF α and EGF receptor transcripts in lesion-only animals, it would be of interest to examine the time course of modulation at time points earlier after onset of the experimental treatment. The slight increase in TGF α mRNA seen in a few animals

immediately around the infusion scar was found in both TGF α - and aCSF-infused striata. Thus, it is probably attributable to continued mechanical injury and gliosis caused by the cannula itself, and not to the infusate.

TGF α infusions dramatically increased EGF receptor mRNA hybridization in the ipsilateral subependymal zone but not in the rest of the striatum. In other tissues, EGF receptor mRNA can be modulated by several chemical and mechanical means. EGF peptide increased EGF receptor mRNA in numerous mammalian cell types *in vitro* (Earp *et al.*, *J. Biol. Chem.* 261:4777-4780, 1986; Kesavan *et al.*, *Oncogene* 5:483-488, 1990). Retinoic acid caused a similar increase in normal rodent fibroblasts (Thompson and Rosner, *J. Biol. Chem.* 264:3230-3234, 1989) and in a transformed rat liver cell line (Raymond *et al.*, *Cell. Growth Diff.* 1:393-399, 1990). Exposure to cyclohexamide, by itself or with EGF, stimulated an increase in cultured human cytotrophoblasts and stabilized EGF receptor transcripts, thus providing a mechanism other than enhanced transcription to increase total abundance of EGF receptor mRNA (Kesavan *et al.*, 1990, *supra*).

Transection of the sciatic nerve in rats brought about a graded increase in EGF receptor mRNA in the severed ends (Toma *et al.*, *J. Neurosci.* 12:2504-2515, 1992). Treatment with protamine increased ¹²⁵I-EGF binding and cell surface receptor number in mouse and human cell lines *in vitro* (Lokeshwar *et al.*, *J. Biol. Chem.* 264:19318-19326, 1989). TGF α may mimic these actions and increase the production and/or longevity of EGF receptor transcripts in extant subependymal cells. It may also stimulate transcription in cells that do not normally express appreciable amounts of EGF receptor mRNA. Both of these effects could be readily investigated further *in vivo* with routine techniques known to those of ordinary skill in the art.

An additional possibility is that TGF α is stimulating proliferation of subependymal cells and increasing the total numbers of cells expressing EGF receptor mRNA. EGF and TGF α are potent mitogens for cultured "EGF responsive" cells explanted from the subependymal region (Reynolds and Weiss, 1992, *Science* 255:1707-1710). The strong inductive effect striatal TGF α infusion had on subependymal EGF receptor mRNA hybridization may indicate that proliferative cells in the intact brain respond similarly to these cell *in vivo*.

3. Combined TGF α infusion and 6-OHDA lesion.

In animals receiving combined lesions and TGF α infusions, striatal TGF α mRNA hybridization was indistinguishable from that in animals receiving combined lesions and aCSF infusions. Although TGF α has potent autostimulatory effects in other tissues, it did not significantly alter the reduction of striatal TGF α mRNA hybridization in the present study. The 6-OHDA-mediated loss of mesencephalic EGF receptor mRNA was similarly unaffected by TGF α infusion. In the latter case, the midbrain lesions were performed, and ipsilateral dopamine cells destroyed, weeks prior to the start of the infusion. Thus, the growth factor would not have had an opportunity to prevent their elimination.

There is some evidence that the dopamine cells themselves express EGF receptor mRNA (Seroogy *et al.*, 1994, *supra*) and that TGF α can moderate the loss of markers for striatal dopaminergic innervation if administered concurrently with the neurotoxin. Therefore, the time interval between the neurotoxic lesions and the administration of TGF α may explain why TGF α infusions had no impact on the abolition of midbrain EGF receptor mRNA.

In human Parkinsonian brains, mesencephalic EGF receptor binding is unchanged from that seen in the normal brain (Villares *et al.*, 1993, *supra*). The huge increases in striatal TGF α (and other neurotrophic factors) with PD (Mogi *et al.*, 1994, *supra*) may mask a reduction in the number of EGF receptor expressing dopamine cells by increasing the levels of expression in the remaining neurons. On the other hand, *in vitro* experiments suggest that many of the trophic effects of TGF α on mesencephalic dopamine neurons are mediated, at least partially, through glia (Alexi and Hefti, 1993, *supra*). TGF α may therefore act through paracrine (direct) and sequential (indirect) modes of transport to influence dopamine neurons.

The pattern of EGF receptor mRNA hybridization in the subependymal zone of TGF α -lesioned animals was similar to that seen in TGF α -nonlesioned animals. The most striking feature in the ipsilateral striata of these animals was a dense ridge of hybridization well out of the body of the striatum, more intense even than the enhanced hybridization in the subependymal zone. The ridge did not correspond to any known anatomical feature and was not evident with the TGF α or TH probes. EGF receptor mRNA hybridization in the non-ridge striatum was the same as in the striata of all other groups.

The neurotoxic damage from the 6-OHDA lesions and the mechanical injury from implantation of the infusion cannula may have stimulated proliferation and activation of glial cells. Previous studies have demonstrated gliosis and increased astrocytic EGF receptor expression as a result of injury (Nieto-Sampedro *et al.*, *Neurosci. Lett.* 91:276-282, 1988; Fernaud-Espinoza *et al.*, *Glia* 8:277-291, 1993). Further, TGF α may play a role in the reactivity of astrocytes (Junier *et al.*, *J. Neurosci.* 14:4206-4216, 1994). Another possibility is that proliferative cells of the subependymal region were drawn away from the ventricle and into the overlying striatum by the combined growth factor infusion and midbrain lesion. TGF α is a potent chemoattractant for diverse cell types (Reneker *et al.*, *Development* 121:1669-1680, 1995), but by itself was not sufficient in most animals to stimulate formation of the ridge. Formation of the cellular ridge may have been facilitated by the midbrain lesions. The origin and identity of these cells will be examined in the following example.

3: Characterization of the Striatal Ridge

As described above, striatal infusions of TGF α , when combined with nigral 6-OHDA lesions, induce the formation of a dense ridge of cells in the body of the striatum that abundantly expresses EGF receptor mRNA but no more TGF α than the surrounding tissue. The ridge was comprised of a mass of densely packed cells, allowing its clear detection using simple thionin staining. The identity of the anomalous striatal ridge was not apparent, but three possibilities were considered.

Gliosis in response to injury is a feature of both traumatic and neurotoxic damage to brain tissue. Typically, both types of brain injury stimulate astrocytosis and infiltration of injured tissue by astrocytes and microglia (Fernaud-Espinoza *et al.*, *Glia* 8:277-291, 1993). Astrocytes have been shown to express EGF receptor immunoreactivity, particularly in response to brain injury (Gomez-Pinilla *et al.*, *Brain Res.* 438:385-390, 1988; Nieto-Sampedro *et al.*, *Neurosci. Lett.* 91:276-282, 1988). In addition, TGF itself stimulates the proliferation of astrocytes (Alexi and Hefti, *Neurosci.* 55:903-918, 1993). Therefore, the possibility that the striatal ridge was a mass of glial cells responding to the combined neurotoxic and mechanical damage and infusion of the growth factor was considered.

A second potential source for the ridge was investigated that was related to the distinctive anatomy of the rodent striatum. The ridge did not correspond to any previously

identified anatomical feature. In rodents, the caudate and putamen are not anatomically distinct structures. No anatomical or neurochemical markers have been identified thus far that distinguish between these two nuclei of the basal ganglia in rodents. However, during prenatal and early postnatal development, neurogenetic gradients within different regions of the developing striatum correspond to characteristic gradients in the caudate and putamen in animals where these nuclei are anatomically discrete (Bayer, *Intl. J. Devel. Neurosci.* 2:163-175, 1984). In rodents, new striatal neurons rostral to the decussation of the anterior commissure are added in a lateral-to-medial gradient such that the latest born neurons are those nearest the lateral ventricle. That same pattern is observed in the development of the caudate nucleus, suggesting that the anterior striatum in rodents is more of a "caudate-like" region. Caudal to the crossing of the anterior commissure, neurons are added in a medial-to-lateral gradient, similar to the developing putamen in animals where it is anatomically distinct. Thus, the posterior rodent striatum may be more "putamen-like". The possibility was considered that the striatal ridge, then, might represent a previously unrecognized border between these two regions of rat striatum that allowed a dense buildup of cells, perhaps due to some neurochemical difference.

A third possibility for the source of the striatal ridge was also examined. Explanted cells from the subependymal zones of the forebrain lateral ventricles of adult mammals have been found capable of proliferating and differentiating into new neurons and glia, particularly when cultured in the presence of EGF-family neurotrophic factors, including TGF α (Reynolds *et al.*, *J. Neurosci.* 12:4565-4574, 1992; Morshead *et al.*, *Neuron* 13:1071-1082, 1994). Recently, EGF or TGF α infused into the lateral ventricle stimulated proliferation of "EGF-responsive" stem and neural progenitor cells in the adult mouse brain (Craig *et al.*, *J. Neurosci.* 16:2649-2658, 1996). A possibility for the source of the striatal ridges in the present studies was that the TGF α infusions stimulated similar proliferative activity in the brains of rats. Additionally, the possibility that the striatal ridges were mass migrations of proliferating neural progenitor cells derived from the subependymal regions was considered.

The experiments described below served to allow characterization of this anomalous striatal ridge using a variety of histochemical and immunohistochemical techniques. The origin of the ridge and the factors influencing its appearance were also

investigated by examining the time course of its formation in the striatum and by altering the combinations of surgical and chemical treatments.

A. Experimental Protocols

Adult male Sprague-Dawley rats weighing 250-300 grams were used throughout the study. Twenty-four animals received standard midstriatal infusions of rat TGF α (0.5 μ g/day; Sigma Chemical Co.). Another 26 rats received either artificial cerebrospinal fluid ("aCSF") or no infusion. A subset of animals in the standard TGF α infusion group and the control group received stereotaxic 6-OHDA injections into the substantia nigra 48 hours after the infusions were begun. Animals used in this portion of the study were classified into six groups according to their infusion-lesion combination, as follows: TGF α infusion, lesion (n=13); TGF α infusion, no lesion (n=11); aCSF infusion, lesion (n=12); aCSF infusion, no lesion (n=9); no infusion, lesion (n=1); no infusion, no lesion (n=4).

Additional animals received TGF α infusions into other regions of striatum, the lateral ventricle, cerebral cortex, or the septum. Four more animals (two per group) received midstriatal TGF α infusions at one-half or one-tenth the standard dose. Also, two animals received midstriatal infusions of epidermal growth factor (EGF) instead of TGF α . The EGF administered in these rats was at the standard 0.5 μ g/day dose. All of the animals in these extra groups were lesioned. Rats in all of the experiments were typically perfused one to 16 days postlesion (3-18 days of infusion). To determine whether the ridge would persist after the infusions ceased, the minipumps were removed from four animals with TGF α infusions at the end of two weeks, but these animals were not perfused until several days later. Brain sections were prepared and stained using various immunocytochemical and histochemical techniques.

B. TGF α Infusion

Rats were anesthetized with 8 mg xylazine and 100 mg ketamine/kg. Infusions of TGF α were provided up to 18 days by Alzet osmotic minipump (2002). The minipumps were filled to about 200 μ l with either aCSF for control animals or 20 μ g TGF α in 400 μ l of aCSF (50 μ g/ml) for experimental animals. Under sterile conditions, the infusion cannula was positioned to stereotaxic coordinates (+1.2 A/P; +2.7 M/L; -6.0 D/V) based on Bregma (Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, Academic

Press, San Diego, 1986) and cemented to the top of the skull with dental cement. The infusate was delivered via cannula at approximately 0.5 μ l/hour. Some additional control animals received infusions either into the lateral ventricle, the overlying cortex, or other areas.

5 C. Neurotoxic Lesion

Forty-eight hours after the minipump implant, rats were anesthetized as above. A chilled 4.8 mg/ml solution of 6-OHDA HCl was prepared immediately prior to injection. Using sterile technique, the neurotoxin was stereotactically injected into the ipsilateral substantia nigra (+3.7 A/P; +2.1 M/L; +2.0 D/V) using interaural zero as a reference (Paxinos and Watson, 1986, *supra*). A 6-8 μ l volume was injected at a rate of 1 μ l/minute.

10 D. Tissue Preparation

Animals were perfused with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) one to 16 days postlesion and their brains placed into 20% sucrose. The next day, the brains were frozen in isopentane at -20°C.

15 Forty-micron coronal sections were then cut on a freezing microtome into 2% paraformaldehyde in 0.1 M PBS. Continuous sections were taken through the striatum and substantia nigra-VTA. Representative sections were taken through the rest of the brain.

E. Nissl Staining

20 Microtomed brain sections for Nissl staining were mounted onto gelatin-coated slides and allowed to dry overnight. They were then dehydrated and rehydrated through ethanol baths, and placed in Thionin solution for approximately four minutes. The sections were dehydrated through the series of ethanol baths, cleared in successive HistoClear™ washes, and coverslipped. Section were viewed under light microscopy and photographed with Technical Pan Film™ (Kodak, Inc.) at ISO 100 (HC-110 processing for six minutes).

25 F. Silver Staining

Degenerating fibers and cells of the ridge were labeled using a modification to the Nauta staining method, similar to Procedure I of Fink-Heimer (Gioli and Karamanlidis, In: Neuroanatomical Research Techniques, R.T. Robertson, Ed., pp. 211-240, Academic Press, New York, 1978). Briefly, free-floating sections were placed into 0.05% potassium permanganate prior to treatment with fresh 1% hydroquinone-1% oxalic acid. They were
30 treated with successive uranyl nitrate/silver nitrate solutions of increasing concentration.

After another rinse, the sections were reacted in ammoniacal silver, then in ethanol/citric acid/paraformaldehyde reducer, and finally in sodium thiosulfate. After staining, the sections were mounted on glass slides and allowed to dry on a slide dryer for 15 minutes. The sections were then dehydrated through successive ethanol washes of increasing concentration, defatted in three successive HistoClear™ washes, and coverslipped.

G. Immunohistochemistry

The quality and the extent of the nigral lesion were determined by the loss of TH-IR in the ipsilateral ventral midbrain. Antibodies against glial fibrillary acidic protein (GFAP), a marker for astrocytes, nestin, a marker for neural progenitor cells, and vimentin, a marker for radial glial cells, were employed in the neurochemical characterization of the ridge. Immunohistochemistry was performed on free-floating sections. Briefly, brain sections were washed in 0.1 M PBS or Tris buffered saline (TBS; 3 x 10 minutes) then incubated for 1 hour in blocking solution consisting of 3% normal goat serum in 0.1 M PBS or TBS with 250 µl Triton X-100. Next, they were incubated overnight at room temperature on a rotator with antibody solution diluted with blocking solution: rabbit anti-TH antiserum (1:500; Eugene Tech Intl., Inc.), rabbit anti-GFAP (1:6400; Dako Corp.), mouse monoclonal anti-vimentin (1:50; Sigma Chemical Co.), or with mouse anti-nestin supernatant (1:20; University of Iowa Hybridoma Bank).

The sections then were washed and incubated for 1 hour with biotinylated goat anti-rabbit antiserum (1:200; Vector Labs, Inc.) for TH or GFAP immunostaining, or biotinylated horse anti-mouse antiserum (Vector Labs, Inc.) for nestin or vimentin immunostaining, then washed and incubated in avidin-biotin complex (ABC Elite kit, Vector Labs, Inc.) for 1 hour. Localization of primary antibody binding was revealed using the diaminobenzidine (DAB) peroxidase technique. The sections were washed thoroughly and mounted on gelatin-subbed slides and allowed to dry overnight. Finally, the sections were dehydrated, cleared, and coverslipped as described above.

None of the animals used in the study displayed adverse effects from the minipump implants or lesion surgeries. All continued to take food and water through the course of the experiments. If the lesion, infusion, or both were not successful, the animals (n=6) were excluded from the initial experimental groups and examined separately. A successful lesion was defined as one that caused complete or near-complete elimination of

ipsilateral nigral TH-IR. A successful striatal infusion was defined as one where the tip of infusion cannula was successfully fixed into the body of the striatum.

As with the *in situ* hybridization studies, all animals receiving intrastriatal TGF α infusions of six days or more displayed a dramatic buildup of cells along the ventricle ipsilateral to the infusion, visible with thionin staining. By comparison, the contralateral striatum showed no such increase, and was indistinguishable from that in aCSF-infused animals. EGF infusions in lesioned animals induced the cellular expansion along the ventricle, but did not induce formation of the striatal ridge. Lower doses of TGF α induced both the cellular expansion and ridge formation, but, qualitatively, the number of cells in each was decreased.

1. Effects of 6-OHDA lesions

None of the lesioned animals receiving artificial cerebrospinal fluid ("aCSF") infusions showed any cellular expansion along the ipsilateral ventricle or any evidence of a striatal ridge. Lesioned animals infused with TGF α did uniformly exhibit the buildup of cells along the ventricle and typically displayed the striatal ridge. Nigral lesions dramatically increased the incidence of formation of the ridge compared to unlesioned animals (Table 1).

2. Morphology and persistence of the ridge

Midstriatal infusion resulted in a characteristic S-shaped ridge arising from the dorsomedial caudate-putamen, sweeping out into the striatum and looping back slightly toward the midline at its ventral end. The dorsal-most portion of the ridge was continuous with the build-up of cells in the subependymal region. Typically, thionin staining was most dense in the dorsal portion of the ridge paralleling the EGF receptor mRNA hybridization. The cellular ridge was generally found throughout most of the rostral-caudal extent of the striatum. The ridge was still prominent in the striatum three months after the TGF α infusion pump was removed.

Infusate	Lesion	Number	Expansion	
CSF	A no	9	0%	0%
CSF	A yes	12	0%	0%

GF α	T no	11	100%	27%
GF α	T yes	13	100%	92%

3. GFAP Immunohistochemistry

Antiserum against glial fibrillary acidic protein (GFAP), a marker for astrocytes, failed to stain cells of the striatal ridge or the cellular expansion along the ventricle. Normal GFAP-IR astrocytic staining was found medial and lateral to the ridge, but was nearly excluded from the ridge itself.

4. Silver staining

Labeling cells non-specifically with a modification of the Nauta method provided additional information about the cells comprising the ventricular cellular expansion and the striatal ridge. One of the most striking features was the huge number of cells making up the subependymal cellular buildup and ridge (Fig. 8). The cells were densely packed and predominantly fusiform in shape (Fig. 8). In the ventral portion of the ridge, elongated cells appeared to stream around fiber bundles of the internal capsule suggesting that the cells were migrating through the striatum.

5. Time course of ridge formation

The density of cells of the ridge allowed us to track its formation using simple thionin staining (Fig. 9). All animals used for the time-course experiment received 6-OHDA lesions and midstriatal TGF α infusions. At time points prior to six days of infusion, there was only a very minor build-up of cells in the subependymal region and no evidence of a striatal ridge. By six days of infusion, there was a clear expansion of cells along the ventricle. At nine days infusion, the ventral portion of the ridge had begun to appear slightly displaced from the ventricle. By 12 days infusion, the ventral portion of the ridge was situated as much as 400 μ m from the ventricle wall. At 16 days infusion, the ridge appeared midstriatum, its ventral portion up to two mm from the ventricle. Thus, the ridge originated in the ventricular region and was increasingly displaced radially in the overlying striatum at greater times of infusion. The estimated difference in distance between the lateral extents of

the ridges and the ventricle wall at 12 days and 16 days of infusion was approximately 1.6 mm.

6. Nestin immunohistochemistry

Monoclonal antibodies against nestin, a marker for neuroepithelial progenitor cells, intensely stained dense collections of fibers throughout the ridge (Fig. 10). No nestin-IR fibers were seen lateral to the ridge, but occasional fibers were observed medial to the ridge. The fibers were oriented primarily orthogonal to the ridge.

7. Alteration of ridge morphology

Lesioned animals infused midstriatally with $TGF\alpha$ uniformly exhibited a characteristic S-shaped striatal ridge in coronal sections. This morphology was dramatically altered in rats with infusions into other areas of the caudate-putamen (Fig. 11). Medial striatal infusions gave rise to an L-shaped ridge near the cannula infusion site with the vertical part of the "L" along the ventricle and the horizontal part extending orthogonally from the ventricle into the striatum. Infusion into the extreme lateral striatum stimulated the formation of a linear ridge parallel to the wall of the lateral ventricle.

8. Vimentin immunohistochemistry

Antiserum recognizing vimentin, a marker for radial glial cells, failed to stain any cells in the striatum, the subependymal zone, or the striatal ridge at two weeks of infusion. However, this result is being investigated further, as the controls were performed in embryonic animals, under conditions that may not ensure elimination of false negatives.

9. Control of ridge position

Compared to the cells of ridges in rats used for *in situ* hybridization experiments, ridge cells in the present series of experiments were maximally displaced much farther from the ventricle (Fig. 12). The difference between these two groups of animals experimentally was the timetable of infusions and lesions.

Animals prepared for *in situ* hybridization experiments received lesions and then underwent a series of behavioral tests starting around the second week postlesion to confirm success of the unilateral lesions. Typically, those animals did not receive infusions until five weeks after the lesion. Thus, the dopamine degenerative process and the striatal infusion of $TGF\alpha$ were temporally separate events.

In the present series of experiments, infusions were begun first; lesions were not performed until 48 hours *after* the infusion pumps were implanted. In these animals, the degeneration of the nigral dopamine neurons, the resulting loss of striatal dopaminergic innervation, and the striatal administration of growth factor were temporally concurrent events.

10. Infusions into brain regions other than the striatum

All rats receiving infusions into brain areas other than the striatum received two-week TGF α infusions and nigral 6-OHDA lesions. Intracerebroventricular (ICV) infusion of growth factor ipsilateral to the lesion stimulated the buildup of cells in the adjacent ventricular wall, but did not induce formation of the striatal ridge in any of the animals.

Septal and some striatal infusions stimulated the formation of septal ridges associated with the medial walls of the lateral ventricles. Septal ridges, like the striatal ridges, were readily detected with EGF receptor mRNA *in situ* hybridization or with thionin staining, but tended to be qualitatively less robust in terms of the density and number of cells.

Dorsal cortical infusions, that is, infusions so shallow that they did not penetrate the corpus callosum, had no discernable effect on cell density along the lateral ventricle. Neither did these dorsal infusions induce formation of a ridge. Cortical infusions in which the corpus callosum was slightly penetrated stimulated expansion of cells along the ipsilateral ventricle, but did not induce formation of a striatal ridge. These animals did exhibit densities of cells in the corpus callosum that might be considered callosal ridges.

H. TGF α Stimulates Cellular Proliferation

The present experiments, together with those described above, demonstrate that TGF α administration was necessary for the formation of the cellular build-up and the striatal ridge. Not a single animal that received a striatal artificial cerebrospinal fluid ("aCSF") infusion - whether lesioned or not - displayed any obvious periventricular cellular expansion when compared to subependymal regions contralateral to the infusions or to normal animals. Clearly, cells of the forebrain are responding to striatal infusion of TGF α by proliferating along the lateral ventricle.

Recent studies with 6-day ICV infusions of EGF or TGF α in mice demonstrated a large increase in the number of cells around the ventricle immunolabeled

with 5-bromo-2'-deoxyuridine (BrdU) or [³H]thymidine, markers for cellular proliferation (Craig *et al.*, *J. Neurosci.* 16:2649-2658, 1996). More than 95% of these cells were also positively immunoreactive for EGF receptor. Cresyl violet Nissl staining also showed an increase in the numbers of cells around the ventricles in these animals in response to growth factor administration.

The possibility that the expanded cell populations along the lateral ventricles were glial cells stimulated by the combined neurotoxic lesion and mechanical injury of the forebrain, and the infused TGF α was considered first. Astrocytes are known to respond to brain injury by proliferating and altering their morphology and functional properties (for review see Norenberg, *J. Neuropath. Exper. Neurol.* 53:213-220, 1994). Additionally, striatal astrocytes possess EGF receptors (Gómez-Pinilla *et al.*, 1988, *supra*; Nieto-Sampedro *et al.*, 1988, *supra*) and are stimulated by TGF α to proliferate (Alexi and Hefti, 1993, *supra*). In the present study, antiserum against glial fibrillary acidic peptide (GFAP), a marker for astrocytes, failed to demonstrate an increase in astrocytes in the ventricular region or in the ridge at two weeks of infusion. In fact, GFAP-IR was largely excluded from these areas. Normal astrocytic staining was seen medial and lateral to the ridge, for instance, but few GFAP-IR fibers were observed within the ridge itself. These findings paralleled those in experiments with six-day ICV infusions of EGF or TGF α : GFAP and an additional marker for astrocytes, S100 β , showed no significant increase around the lateral ventricle (Craig *et al.*, 1996, *supra*). Vimentin can also be expressed by reactive astrocytes (Federoff *et al.*, *J. Neurosci. Res.* 12:14-27, 1984), but vimentin-IR was not observed in any of the sections examined. Markers for microglia (MAC-1) and oligodendrocytes (MAG, CNP, O4 and Rip) also did not change significantly (Craig *et al.*, 1996, *supra*). Thus, the immunohistochemical evidence demonstrated that the TGF α -induced expansion of cells along the ventricle and in the striatal ridge were not the result of gliosis.

1. Cellular morphology and orientation

Silver and thionin staining clearly reveal the huge numbers of cells within the cellular aggregation along the ventricle. The cells were most dense and most numerous in the dorsal portions of the subependymal zone and ridge. The cells were predominantly fusiform, similar to migrating neural progenitor cells in the developing brain, with their long axes oriented orthogonal to the ventricle wall (or to the dorsolateral extension of the subependymal zone bordering the dorsal striatum). Silver stained cells in the ventral segment of the ridge appeared to stream around fiber bundles of the internal capsule, suggesting that they were migrating through the striatum.

To determine whether the cells were indeed migrating, a time course experiment was performed to examine the development of the ridge and its location as a function of time after the start of the growth factor infusion.

2. Migration of cells of the striatal ridge

The progressive expansion of cells along the lateral ventricle and the subsequent radial movement of these cells as a dense ridge proved that the cells were indeed migrating *en masse* through the striatum. As such, the ridge could not have been an anatomical delineation between the rodent putative "caudate-like" and "putamen-like" regions of striatum.

3. Stimulation of neural precursor cells

Although none of the immunomarkers for mature astrocytes, neurons, microglia, or oligodendrocytes labeled cells of the periventricular expansion or the striatal ridge, monoclonal antibodies recognizing nestin, an intermediate filament expressed by neuroepithelial precursor cells, intensely stained cell processes in the ridge and along the ventricle. Reactive astrocytes can also express nestin-IR, but the negative GFAP-IR in cells of the ridge eliminated the possibility that astrocytes formed a significant portion of the ridge cells. Nestin-IR has been used in recent years to identify and label neural precursor cells *in vitro* and *in vivo* (Lendahl *et al.*, *Cell* 60:585-595, 1990; Craig *et al.*, 1996, *supra*). The strong nestin-IR in the striatal ridge and the lack of immunostaining for glial markers support the conclusion that the cells of the ridge are predominantly neural progenitor cells.

Thus far, two distinct cell populations have been identified in adult mammalian brain that can give rise to new neurons and glia (Morshead *et al.*, 1994, *supra*). One, the relatively quiescent cell population, are believed to be true multipotent neural stem

cells. The other, the constitutively proliferating population, are believed to be neural progenitor cells, descendent from the stem cells. The stem cell population is thought to remain in the ependymal or subependymal zone and replenish the progenitor cell population as they die or migrate away. The term "neural precursor" is used here to describe any undifferentiated proliferative cell capable of giving rise to neurons and glia in the adult mammalian brain, whether these cells are neural stem cells or neural progenitors.

Previous studies have shown that many neural progenitor cells die in the subependymal zone before they can migrate from the region (Morshead and Van der Kooy, *J. Neurosci.* 12:249-256, 1992). However, it was recently discovered that many others indeed survive and migrate along a highly-restricted path to the olfactory bulbs where they differentiate into olfactory interneurons (Luskin, *Neuron* 11:173-189, 1993; Lois and Alvarez-Buylla, *Science* 264:1145-1148, 1994). They migrate tangentially along the wall of the lateral ventricle in a process called "chain migration" wherein chains of migrating cells are ensheathed by specialized GFAP-IR astrocytes (Rousselot *et al.*, 1994; Lois *et al.*, *Science* 271:978-981, 1996).

The subependymal zone along the forebrain lateral ventricles, then, is much more than a dormant remnant of the embryonic neuroepithelium. In normal unmanipulated brains, it continues to give rise to new neuroblasts that migrate rostrally and differentiate into neurons. Under the influence of EGF-family neurotrophic factors, including TGF α , subependymal neural precursors can be stimulated *in vitro* to give rise to large numbers of new neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, *Science* 255:1707-1710, 1992). From these explant studies, it became clear that the highest concentrations of neural precursor cells were found in the dorsal portion of the subependymal zone, along the dorsal border of the caudate-putamen.

There is even some recent evidence that neural precursors may be stimulated to increase their numbers and produce new neurons and glia *in vivo* (Craig *et al.*, 1996, supra). Cells double-labeled with BrdU and markers for mature neurons and glia were found diffusely distributed throughout the striatum, septum, and cortex after six days of ICV infusion of EGF and up to seven weeks of post-infusion survival. However, no mass migration of subependymal cells into the adjacent striatum was observed in that study (in marked contrast to the results obtained by the present method) and the numbers of cells were

quite modest compared to the densely-packed mass of cells observed in the striatal ridge described herein. Moreover, none of the animals in Craig *et al.* received an infusion sufficient to stimulate the mass migration presently observed and none of the animals in that study received nigral 6-OHDA lesions, which are shown for the first time herein to dramatically increase the incidence of migration.

4. Evidence of neuronal phenotype

A question that remained was whether the cells within the massive expansion along the ventricle and the migrating striatal ridge truly were neural progenitor cells. Table 2 summarizes the data supporting the conclusion that these cells are indeed neural progenitors.

The neurochemical evidence showed that they do not express markers for mature neurons, astrocytes, oligodendrocytes, or microglia. They did, however, intensely express a marker for immature neural progenitor cells. They expressed markers for cellular proliferation.

They arose from the wall of the lateral ventricle where neural precursors are located in the adult rodent brain, expanded laterally and migrated radially away from the ventricle.

Furthermore, their cellular morphology was fusiform and their processes were oriented orthogonal to the ventricle and the ridge, similar to migrating neural progenitors in the embryonic brain and consistent with their migration from the subependymal region. The data summarized in the following Table (Table 2) indicates that cells of the periventricular expansion and the striatal ridge are neural progenitors arising from subependymal neural stem cells.

Evidence of Progenitor Phenotype for Cells of the Expansion and Ridge	
Neurochemical	
Express abundant EGF receptor mRNA* and immunoreactivity	
Immunonegative for GFAP* or S-100 β markers for astrocytes	
Immunonegative for NeuN, a marker for mature neurons	
Immunonegative for MAG, CNP, 04, or Rip, markers for oligodendrocytes	
Immunonegative for vimentin*, a marker for radial glia	
Immunonegative for MAC-1, a marker for microglia	
Immunopositive for nestin*, a marker for neuroepithelial precursors	
Morphological	
Elongated somata in ridge oriented orthogonal to subependymal zone	
Nestin-IR processes in ridge oriented normal to subependymal zone	
Anatomical	
Arise from the subependymal zone	
Highest density of cells is in the dorsal subependymal region	
Physiological	
Respond to TGF α administration by increasing their numbers	

5. Mechanisms of migration

The mass migration of these cells into the striatum could be controlled by striatal dopamine denervation and by the location of the infusion cannula, but the mechanism of migration was unclear. The fact that the shape of the ridge could be modified simply by changing the site of infusion initially suggested a chemoattractant effect. TGF α is known to be a potent chemoattractive agent for diverse cell types (Ju *et al.*, *J. Invest. Dermatol.* 100:628-632, 1993; Panagakos, *Biochem. Mol. Biol. Int.* 33:643-650, 1994). Its abundant expression in the perinatal caudate-putamen may indicate that it performs a similar role in the development of the striatum.

Neural precursor cells in the normal brain are located in a thin region in the wall of the lateral ventricle. Infusions into the mid-striatum were closer to cells of the dorsal end of this region. Presumably, cells migrating into the striatum would move toward putative higher concentrations of growth factor at the tip of the infusion cannula where the TGF α was released. The characteristic S-shape of ridges in animals with mid-striatal infusions might have resulted from receptor saturation of cells in the dorsal segment of the subependymal zone nearest the tip of the infusion cannula. Cells with saturated EGF receptors might have halted their migration once they moved close to the infusion site. Cells near the ventral end of the subependymal zone would see a lower putative growth factor concentration and would have to travel farther toward the infusion site before the concentration of TGF α increased enough to saturate their receptors. This differential migration with receptor saturation could explain the characteristic S-shape of these ridges.

Infusions into the medial striatum resulted in L-shaped ridges, again in keeping with a neurochemical gradient/receptor saturation effect. In this instance, dorsal subependymal cells may have had their receptors saturated and their migration halted before they could even emerge from the subependymal zone. Only cells in the most ventral portion of the subependymal region could migrate away from the ventricle.

Extreme lateral infusions essentially would have presented a similar putative concentration gradient to cells along most of the length of the proliferative region. The subependymal cells all migrated a similar distance from the ventricle, resulting in a roughly linear ridge, consistent with the idea of a chemoattractant, neurochemical gradient effect.

However, immunohistochemical evidence from the characterization studies cast doubt on the idea that a simple chemoattractant effect could entirely explain the mass radial migration. Nestin-IR processes of the migrating cells were not aligned with the tip of the infusion cannula, the region of the putative highest concentration of growth factor. Instead, they were oriented normal to the ventricle and the ridge. This orientation suggested that the cells migrated orthogonally into the caudate-putamen -- as migrating neural progenitors do from the embryonic striatal neuroepithelium -- not obliquely toward the tip of the infusion cannula.

Two additional findings discounted the role of simple chemoattraction in the migration of the ridge. First, the cells did not begin to migrate as they were produced; they

increased their numbers along the ventricle for a period of more than a week, then migrated *en masse* as a dense sheet of cells. Further, lesion of the ipsilateral substantia nigra greatly increased the incidence of migration. Both of these observations suggested a more complex set of factors influencing the migration of the cells. These data did not entirely rule out a role for chemoattraction in the migration of the ridge, but they did indicate that a simple chemoattractive effect could not by itself account for it.

Another mechanism that might have contributed to the radial migration of neural progenitors in the adult striatum was the reconstruction of the radial glial scaffold due to the neurotoxic lesion, the mechanical injury done by the surgical implantation of the infusion cannula, or both. Radial glia guide the migration of neural progenitor cells in many regions of developing brain. They are anchored along the ventricle and extend their processes radially into the overlying parenchyma. They normally are transformed into GFAP-IR astrocytes in the early postnatal period once neuroblast migration is complete, and cease expression of vimentin and nestin.

Freezing injury of the cortical plate in neonatal rats inhibited radial glial transformation and caused the persistence of glial expression of vimentin and nestin in the injured regions of the adult brain (Rosen *et al.*, *Dev. Brain Res.* 82:127-135, 1994). Kainate lesion of the adult rat hippocampus induced radial glial morphology and expression of nestin-IR and vimentin-IR in astrocytes of the hippocampal subependymal zone, suggesting that brain injury could stimulate a reversion of astrocytic phenotype to one found in the embryonic brain (Clarke *et al.*, *Neuroreport* 5:1885-1888, 1994).

The present immunohistochemical experiments do not provide support for this phenomenon. Nestin-IR fibers were found in abundance in radially-oriented fibers along the ventricle at infusion day nine, but at later time points, they no longer remained along the ventricle. As the cells of the ridge migrated away from the subependymal region, so did the nestin-IR fibers. Furthermore, immunostaining for vimentin, a specific marker for radial glia, did not reveal any labeled fibers either in the ridge or in the subependymal region. Thus, it is unlikely that any astrocytes were reverted to their embryonic radial glial phenotypes or that astrocytic reversion played a role in the radial migration of the neural progenitor cells.

A newly-described mode of migration employed specifically by neural progenitors in the adult mammalian brain elucidates the tangential movement of these cells from the forebrain subependymal zones to the olfactory bulbs (Lois *et al.*, 1996, *supra*). Rostrally migrating neuroblasts are densely packed and sheathed by GFAP-IR astrocytes bordering their highly-restricted migratory pathway. The migrating cells essentially form a solid stream of moving cells within a tube of specialized glial guide cells. The neural precursors in our experiments migrated as a sheet through the striatal neuropil -- not along a restricted path -- and were not associated with GFAP-IR cells. In fact, the proliferative subependymal zone and the cellular ridge largely excluded GFAP-IR. Thus, the mechanism of chain migration could not account for the radial migration seen in the present studies.

Another mechanism possibly underlying the mass neural progenitor migration was that cells of the striatum may have altered their expression of growth factors, cell adhesion molecules, or other substances in response to injury. In this scenario, the striatum may have been stimulated to provide its own chemoattractants or molecules that facilitate radial migration. Alternatively, it may also have been induced to downregulate expression of substances that inhibit migration.

Recent studies examining cell adhesion molecules in the striatum and subependymal region provide particularly intriguing insight. Highly polysialylated neural cell adhesion molecule (PSA-N-CAM) immunoreactivity is intensely expressed in the developing rodent striatum, but decreases as the animal matures (Aaron and Chesselet, *Neurosci.* 28:701-710, 1989; Szele *et al.*, *Neurosci.* 60:133-144, 1994). PSA-N-CAM expression, however, persists along the adult forebrain subependymal region (Rousselot *et al.*, 1994; Szele *et al.*, 1994, *supra*). Partial decortication-induced striatal deafferentation dramatically increased expression of PSA-N-CAM and another adhesion molecule, L1, in the subependymal zone (Poltorak *et al.*, *J. Neurosci.* 13:2217-2229, 1993; Szele and Chesselet, *J. Comp. Neurol.* 368:439-454, 1996). In the human brain, PSA-N-CAM expression is low in normal striatum, but is increased in the striata of Huntington's disease patients, particularly in the subependymal zone (Nihei and Kowall, *Ann. Neurol.* 31:59-63, 1992).

Of special interest were the changes occurring in fibronectin mRNA expression in the striatum after partial unilateral frontal decortication (Popa-Wagner *et al.*, *Neuroreport* 3:853-856, 1992). Fibronectin is one of a number of molecules that have been

shown to support neural migration *in vitro* (Fishman and Hatten, *J. Neurosci.* 13:3485-3495, 1993). Fibronectin mRNA hybridization was increased to a maximum level at 72 hours in the portion of the striatum immediately under the wound cavity. This early increase was interpreted as a component of the short-term wound healing process. Fibronectin expression in the greater ipsilateral striatum followed a longer-term increase, peaking at about ten days post lesion. This secondary increase was interpreted as the striatal response to deafferentation. Increases in expression of two other mRNAs that code for N-CAM and alpha tubulin followed only the early wound healing-related spatial and temporal patterns.

The ten-day peak of striatal fibronectin mRNA expression after deafferentation corresponds well to the delay of ridge migration following striatal dopamine denervation in the present studies. The delay of peak fibronectin mRNA expression may help explain why the progenitor cells of the invention did not begin to migrate radially until around the ninth day of infusion, and why, when they finally did migrate, they migrated *en masse*. In animals where the infusions and lesions were separated by several weeks, the maximum lateral migration distances at two weeks of infusion were dramatically reduced. This observation, too, is consistent with the transient peak in striatal fibronectin expression. In the few animals with ridges that did not also receive nigral lesions, mechanical injury of the overlying cortex may have stimulated enough fibronectin expression in the striatum to facilitate the migration. Fibronectin, then, may be upregulated in response to dopamine denervation of the ipsilateral striatum and, in turn, may temporarily facilitate radial migration of neural progenitors from the subependymal zone.

Another possible influence on radial migration of neural progenitors in the adult striatum may stem from a secondary effect by cell adhesion molecules. Infusion of N-CAM into the brains of adult rats receiving stab wounds to various areas of the brain, including striatum, inhibited astrocytic proliferation (Krushel *et al.*, *Proc. Natl. Acad. Sci. USA* 92:4323-4327, 1995). Astrocytes release factors inhibiting neurite outgrowth and may thus inhibit neural regenerative responses. Thus, denervation of the striatum, and the associated increase in subependymal PSA-N-CAM may release inhibition of neural regeneration.

The enhancement of the migration effect in dopamine-denervated striatum may also have been related directly to the loss of dopaminergic innervation. During

embryonic development of the striatum, immature neurons originate in the ventricular region and migrate radially (Bayer, 1984, *supra*; Bayer and Altman, *Prog. Neurobiol.* 29:57-106, 1987). The developmental migration of striatal neurons takes place prior to dopamine innervation by afferents from the midbrain. Stimulation of D₂ dopamine receptors on hypothalamic neurons dramatically attenuated TGF α mRNA expression and pituitary growth (Borgundvaag *et al.*, *Endocrinology* 130:3453-3458, 1992). Although dopamine receptor-mediated inhibition of TGF α expression has not been studied in the subependymal zone, it is consistent with the depressed incidence of migration in non-lesioned animals. Thus, dopamine innervation during development may inhibit migration of striatal cells as the forebrain dopaminergic innervation becomes established.

Striatal dopamine may also contribute to the downregulation of striatal TGF α early in postnatal development as dopaminergic afferents become established. Dopamine denervation of the adult striatum may mimic for striatal cells some of the local chemical environmental cues normally only seen in the developing striatum -- for instance, a reduction of available ligand for dopamine receptors expressed on striatal neurons. Striatal dopamine innervation has also been linked in a reciprocal manner to expression of extracellular matrix (ECM) molecules by astrocytes (Gates *et al.*, *J. Chem. Neuroanat.* 6:179-189, 1993). Interestingly, TGF α is selectively elevated in the striata of PD patients (Mogi *et al.*, *Neurosci. Lett.* 180:147-150, 1994) similar to the elevated expression in the embryonic striatum. If striatal TGF α is regulated by dopamine innervation, this increase may relate to the reduction of striatal dopamine and the consequent release of inhibition of TGF α expression.

Whatever the underlying mechanism, the time course experiment proved that subependymal cells could be stimulated to increase their numbers and migrate radially *en masse* into the adjacent striatum in the adult rat brain. Experiments in which the location or the dose of TGF α infusion was varied showed that movement of the striatal ridge and the gross numbers of cells involved could be controlled. The characterization experiments provided abundant evidence that the subependymal cellular expansion and the dense striatal ridge were composed of neural progenitor cells. The importance of these discoveries is discussed below, together with their potential application for the treatment of human neurodegenerative disease and traumatic brain injury.

Example 3: TGF α Infusion Stimulates *In vivo* Induction of Massive Proliferation, Directed Migration and Differentiation of Neural and Glial Cells in the Adult Mammal Brain

The following example describes studies that demonstrate that methods and compositions of the invention can be used to induce the proliferation, directed migration and differentiation of neural and glial cells in the adult mammalian brain. In particular, these studies demonstrate the *in vivo* induction of massive proliferation, directed migration and differentiation in the forebrain of the adult rat following unilateral 6-hydroxydopamine (6-OHDA) lesioning of the substantia nigra dopaminergic neurons, and infusions of TGF α into ipsilateral forebrain structures.

Adult male Sprague-Dawley albino rats (240-350 g) (n=130) were obtained from Simonsen Labs (Gilroy, CA). The animals were maintained in a temperature and humidity controlled campus vivarium. All aspects of animal handling and surgery were in accordance with current NIH guidelines and UCI Institutional Animal Care and Use Committee protocols.

6-Hydroxydopamine ("6OHDA") and transforming growth factor alpha infusions were administered as follows: *6-Hydroxydopamine lesions*: Animals were anesthetized with intraperitoneal Xylazine (8 mg kg⁻¹) and Ketamine (100 mg kg⁻¹) (Western Medical, Arcadia). A chilled sterile solution of 1.0-4.8 mg/ml of 6-hydroxydopamine HCl (6-OHDA) (Sigma, Inc.) in 0.9% saline with 0.01% ascorbic acid was prepared immediately before injection. The animals were immobilized in a Kopf stereotaxic, and placed on a warm mat at 37°C. Using aseptic techniques, 2.5-8 μ l of 6-OHDA solution was stereotactically injected at the rostral border of the substantia nigra-ventral tegmental area (+3.7 A/P; +2.1 M/L; +2.0 D/V) at a rate of 1 μ l min⁻¹ using interaural zero as a reference. The total duration of surgery was approximately 45 minutes. *Growth factor infusions*: Osmotic minipumps (Models 2002 and 2004, Alzet, Inc.) were used for transforming growth factor alpha (TGF α) and artificial cerebrospinal fluid ("aCSF") infusions. They were implanted at a predetermined period contemporaneous with, or after, the 6-OHDA lesion. The minipumps were filled with approximately 200 μ l of a solution containing 10, 50, or 100 μ g of TGF α (Stem Cell Pharmaceuticals, Seattle, WA) in aCSF for experimental animals or plain aCSF for control animals, and incubated overnight in normal saline at 37°C prior to implantation. Under aseptic conditions, the 5 mm cannula attached to the minipump (Brain Infusion Kit, Alzet, Inc.) was stereotactically implanted into the left caudate-putamen (+1.2 A/P; +2.7 M/L) using Bregma as a reference. The minipump was placed

subcutaneously in the interscapular region. The infusate was delivered directly into the striatum by the minipump at a rate of approximately 0.5 μ l h⁻¹. The animals (n=130) were subdivided into groups receiving simultaneous infusions (TGF α - n=25, aCSF- n=13), infusions two weeks after 6- OHDA (TGF α - n=20, aCSF-n=11), controls receiving infusions without a 6-OHDA lesion (TGF α - n=8, aCSF- n=8), intraventricular infusion with 6-OHDA lesion (n=2) or 6OHDA lesion without any infusion (n=43).

Behavior testing: The apomorphine-induced rotation technique was used to test behavioral changes in both the 6-OHDA lesioned and non-lesioned animals. A fresh solution of apomorphine (0.25mg/ml) was prepared each time. The animal was set on a rotometer bowl and its behavior observed for five minutes. It then received a subcutaneous injection of apomorphine (2.5mg/kg body weight) and was placed back in its cage and allowed to rest for 10minutes. Apomorphine-induced rotations were thereafter observed at ten-minute intervals, each session lasting five minutes for a total of 15 minutes.

In situ hybridization histochemistry: TGF α mRNA probes were generated from a 550 nucleotide XbaI/BamHI cDNA fragment from the 5' end of rat TGF α , subcloned into pGEM 7Zf (Promega, Inc). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor mRNA probes were produced from a 718 base pair BamHI/SphI insert from the 5' end of the gene in pGEM 7Zf. Probes for rat TH were created using the 1.3 kD BamHI/EcoRI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of [35S] UTP (NEN Research, Inc). *In situ* nucleic acid hybridization was performed according to Simmons (1989) J. Histochem. 12:169-181). Parallel sections from experimental and control animals were hybridized overnight at 65°C with sense or antisense probes at a concentration of 107 c.p.m. /ml. Adjacent sections from the same animals were hybridized to each of the probes so that direct comparison could be made of their anatomical distributions. Slides from experimental and control animals were grouped together and apposed with [14C]-labeled brain paste standards to autoradiographic Beta Max Hyperfilm™ (Amersham) for 3-7 days. After successful development of the autoradiography film, analysis and quantitation was done using MCID (Imaging Research, Inc). Densitometry readings were sampled at multiple sites within each anatomical region of interest and averaged. Relative

concentrations of TGF α and EGF receptor from the hybridization process were then estimated using a computer-generated third degree polynomial standard curve constructed from the [14C] brain paste standards. The estimated values for each region in each treatment group were then averaged and their standard errors calculated. Brain regions ipsilateral to the experimental treatments were compared to the corresponding regions in control brains at approximately the same positions. Significance of the comparisons was determined using the Student's t-test.

BrdU administration: Animals received 50mg/kg 5'-bromo-2-deoxyuridine (BrdU, Boehringer-Mannheim) intraperitoneally 12 hourly for 3 days from the day of surgery.

Tissue preparation: Animals were sacrificed at timed intervals between 1 and 28 days by either decapitation or perfusion. After decapitation the brains were quickly removed and frozen in isopentane at 20°C. Coronal cryostat sections were cut at 20 μ m and thaw adhered to Vectabond™-(Vector labs, Inc) coated slides. The sections were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour, rinsed in phosphate buffer and air dried, then stored at -20°C until processed. After cardiac perfusion first with saline then 4% paraformaldehyde, the brains were quickly removed, and post-fixed in 4% paraformaldehyde for an hour, then cryoprotected in 30% sucrose overnight at 4°C. The brains were then cooled and cut at -20°C on a freezing microtome.

Immunohistochemistry: Perfused brain tissue was cut on a freezing microtome at 40 μ m and free-floating sections placed in 0.1M phosphate-buffered saline (PBS), pH 7.4. Blocking was done using 10% normal goat serum with 0.4% Triton X in PBS for one hour, prior to primary antibody incubation. Primary antibodies were dissolved in blocking solution as follows: Rabbit anti-GFAP (1:6400, Dako Corp.), mouse anti-b-III tubulin (1:100, R&D systems), rabbit anti-Tyrosine Hydroxylase (1:2000, Sigma), rabbit anti-s100B (1:2500, R&D Systems), and mouse anti-Nestin (1:20, Hybridoma Bank), mouse anti-doublecortin at 1:250, rat anti-dopamine transporter (1:5,000; Chemicon). The tissues were then incubated overnight, at room temperature in primary antibody, then rinsed in PBS and incubated in biotinylated secondary antibodies (anti-mouse, anti-rabbit) in blocking solution. Tissues were rinsed in PBS and incubated in ABC solution (Vector ABC Elite) for 1 hour, then visualized using DAB-Peroxidase histochemistry (Sigma). For BrdU immunocytochemistry, DNA was denatured using 50% formamide in 2X SSC for 2 hours at 65°C, and tissue sections were then rinsed in 2X SSC for 5 minutes and incubated in 2M HCl at 37°C for 30 minutes. The tissue slices were

then rinsed in 0.1 M boric acid pH 8.5 for 10 mins, followed by a 5-minute rinse in PBS.

Immunocytochemical staining for BrdU was then carried out as above.

Silver staining for cellular morphology: Cells were labeled using a modification to the Nauta method, similar to procedure 1 of Fink-Heimer (R.A. Giolli and A.N.

Karamanlidis, Neuroanatomical research techniques. Academic press, New York, 1978. pp.211-240). Free floating sections were placed into 0.05% potassium permanganate prior to treatment with fresh 1% hydroquinone/1% Oxalic acid, then treated with successive uranyl nitrate /silver nitrate solutions of increasing concentration. After another rinse the sections were reacted in ammonical silver, then in ethanol/citric acid/paraformaldehyde reducer, and finally in sodium thiosulphate. After staining, sections were mounted on glass slides, dehydrated and cover-slipped.

Morphological studies of proliferation, directed migration and differentiation induction: Conditions sufficient to induce the massive proliferation of multipotential stem cells originating in the subventricular zone (SVZ) and the subsequent directed migration of these neuroprogenitor cells into the striatum was first determined. Combinations of infusions of TGF alpha into the ipsilateral striatum, and 6-OHDA injections into the ipsilateral substantia nigra-ventral tegmental area (SN-VTA) was used. Since TGF alpha-responsive stem and neuroprogenitor cells in the SVZ have been shown to express epidermal growth factor receptor (EGFr) mRNA, *in situ* nucleic acid hybridization and other anatomical and behavioral techniques was used to demonstrate TGF alpha-responsiveness in the SVZ. In animals receiving 6-OHDA lesions of SN-VTA and artificial cerebrospinal fluid (aCSF) control infusions into the striatum, there was no significant change in the expression of EGFr mRNA in the SVZ. In a second control group receiving TGF alpha infusions, but no 6-OHDA lesions, there was a significant increase in the expression of EGFr mRNA in the SVZ for the duration of the infusion. In the experimental group receiving both a 6-OHDA lesion and a TGF alpha infusion, there was also a significant increase of EGFr mRNA expression in the SVZ. In animals that received more than nine days of TGF alpha infusion, there was also an additional ridge (or thick layered sheet in three dimensions) of EGFr mRNA-positive cells encroaching into the striatum from the SVZ. This ridge was most pronounced in animals, which received concurrent 6-OHDA lesion, and 10, 50 or 100 µg of TGF alpha infusion, but also was present in animals receiving TGF alpha infusions starting 14 days after the 6-OHDA lesion. Both the 50

and 100 ug dose appeared to be equally effective, and the 10 ug dose was minimally less effective in inducing ridge formation.

In a serial time-course analysis, after 4 to 6 days of continuous TGF alpha infusion, in lesioned animals, there was a pronounced thickening of the SVZ. By nine days, however, the ridge separates, *en masse*, from the SVZ, and at later time periods from 14 to 21 days, appearing progressively lateral to the SVZ towards the site of the TGF α infusion. The exact shape and apparent movement of the ridge depends on the site of the TGF alpha infusion. For example, a ventral striatal infusion results in an S-shaped ridge, whereas a dorsal striatal infusion resulted in a shorter and thicker ridge mass adjacent to the dorsal SVZ. The ridge of cells is observed whether the TGF alpha infusion is started concurrent with the lesion, two days prior to, or 14 days after the lesion.

In order to determine if this directed migration is limited to striatal targets, infusions of TGF alpha were made into the septum. In these cases, there was a massive proliferation of the SVZ and a subsequent ridge appeared medially, in the septum toward the TGF alpha infusion site. Additionally, in some cases, a smaller ridge of cells could also be seen on the medial side of the contralateral SVZ in the septum, or migrating through the corpus callosum. In the cases where there was an infusion of TGF alpha into the lateral ventricle in 6-OHDA-lesioned animals, there was a temporary proliferation of SVZ cells but there was no migratory ridge.

Characterization of the ridge: To further characterize the cells in the TGF alpha-induced ridge, a series of morphological and immunocytochemical techniques was performed. Silver staining revealed fusiform cells in the ridge, oriented orthogonal to the most adjacent area of the SVZ. This is consistent with the possibility that these are cells are migrating from the SVZ in parallel. Following TGF alpha infusion into the brain, systemically administered 5'BrdU was incorporated into the SVZ with a specific and massively increased incorporation in the first three days. Subsequently, 5'BrdU positive cells were seen in the ridge, striatum, external capsule and cortex adjacent to the infusion cannula, indicating that the ridge cells are recently generated *de novo*.

To determine the lineage and differentiation of the cells in and around the TGF alpha-induced ridge, the samples were first stained for nestin, a cytoskeletal marker for early lineage neuronal and glial progenitors. The SVZ and ridge cells were nestin-positive from the

fourth day of TGF alpha infusion. In order to determine if the ridge cells are of restricted glial and/or neuronal lineages, we stained for s100 β (astrocytic lineage) and β -III tubulin (neuronal lineage). At 1, 4, 7 and 9 days of TGF alpha infusion, there was no evidence of significant positive staining of these markers, but starting at 14 days, β -III tubulin positive cells were seen. s100 β positive cells were also seen in and around the ridge. Staining for these restricted astrocytic and neuronal lineage markers was, however, greatly reduced by 28 days.

To determine if TGF alpha infusions in these same 6-OHDA-lesioned animals resulted in the new appearance of differentiated neurons in the striatum, tyrosine hydroxylase (TH) (potentially dopamine releasing) immunoreactivity was carried out. Within the second to third week of infusion, occasional TH positive cells were seen near the infusion cannula. From the third and fourth week of infusion, increasing numbers of TH positive cells were seen in the striatum. Immuno-fluorescence staining was also carried out to verify that the ridge is densely populated with BrdU positive cells, and that large clusters of beta-III tubulin positive neuronal precursors are present in the BrdU rich ridge. Doublecortin (dcx) (see, e.g., Chelly (1998) Mol. Psychiatry 3:290-292; a marker for migrating young neurons, is also present in the ridge cells.

Recovery of function: To determine whether there is a functional correlate of the repopulation of neural cells in the striatum observed in these morphological and immunocytochemical studies, two sets of behavioral tests were carried out in some of these animals (see Table 1). One group received a unilateral 6-OHDA lesion of the SN-VTA and fourteen days later, they received a 14-day continuous infusion of either aCSF or TGF alpha into the ipsilateral striatum. Rotational behavior in response to systemically administered apomorphine was tested prior to the lesion, and subsequently weekly for four weeks, both prior to and following the TGF α /aCSF infusions. In this rodent model of Parkinson's disease, there was a statistically significant ($p < .05$, two-tailed t-test) 38% improvement in the rotational behavior of the TGF α versus aCSF-treated control animals.

Table 1 summarizes data from behavioral experiments using a Parkinson's Disease model. Apomorphine-induced rotations in 6OHDA lesioned animals, and 14 days later infused for 14 days with aCSF (control) or TGF α (experimental group). The order of animals in the table is by descending order of % change of rotation. The % improvement of rotational behavior (3 five minute periods) between the TGF α treated animals and aCSF treated animals was 38% (significant at $p < .05$, two tailed t-test, unequal variance)

TABLE 1

TGF α	<u>Parkinson's model infusions</u>		
	Pre-infusion	Post-infusion	% change
1	66.5	14.5	-78.2
2	90.0	22.0	-75.6
3	48.0	14.5	-69.8
4	60.0	25.0	-58.3
5	60.0	40.0	-33.3
6	58.5	39.5	-32.5
7	163.5	140.5	-14.1
8	50.0	49.0	-2.0
9	33.0	33.0	0.0
10	37.0	47.0	27.0
11	103.5	185.7	79.4
MEAN	70.0	55.5	-23.4
SDEV	37.4	55.4	48.5

aCSF	Preinfusion	Postinfusion	% change
1	66.0	45.0	-31.8
2	158.5	151.0	-4.7
3	42.0	41.0	-2.4
4	40.0	40.0	0.0
5	71.0	71.0	0.0
6	94.0	96.0	2.1
7	38.0	41.0	7.9
8	144.5	158.5	9.7
9	63.5	100.5	58.3
10	101.0	162.0	60.4
11	84.0	138.0	64.3
MEAN	82.0	94.9	14.9
SDEV	40.4	50.5	31.5
TTEST	(two tailed, unequal variance)		0.04

A separate group of animals (individual animal data not shown) received TGF α , aCSF or no infusions starting contemporaneously with the 6-OHDA lesion, as opposed to two weeks following the 6-OHDA lesion. In the groups that received either no infusion or an aCSF infusion, contralateral rotational behavior in response to an apomorphine injection was observed at each of 3-4 week intervals following the day when the animals received both the 6-OHDA injection and either aCSF infusion or no striatal infusion at all. In contrast, 10 of the 11 TGF α -treated animals did not exhibit rotational behavioral asymmetry following apomorphine injections during the entire four-week period. Other motor behaviors such as exploratory

behavior in the cage and consummatory behavior, on the other hand, did not appear to be adversely affected in the TGF α -treated animals.

In summary, these findings provide the first evidence of induced coordinated proliferation, directed migration *en masse* and phenotypic differentiation into TH-positive neurons (potentially dopamine releasing) of neural stem cells and their progenitors in the mammalian CNS *in vivo*. The parallel morphological and behavioral rotation experiments demonstrate the usefulness of exogenous TGF α administration in the amelioration and reversal of symptoms of Parkinson's Disease and other neurodegenerative disorders, and acute CNS injury, for example, due to trauma and stroke. These studies also demonstrate that the combination of TGF α infusion and endogenous stimuli arising from the injury signals in experimental animals resulted in a massive proliferation of the cells of the SVZ, followed by a directed migration *en masse*, towards TGF α infusion sites in the striatum, septum, and external capsule or cortex. Further, a progressive pattern of proliferation, migration, maturation and differentiation of newly generated cells was demonstrated; this lead to the generation of cells with neuronal and glial phenotypes. These TGF α - induced cells also differentiated into cells having the phenotypic specialization of some neurons. These studies also demonstrated that this exemplary method of the invention had the desirable functional result, that is, reversal of motor dysfunction.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.